ICSI Children

Follow-up after ICSI with ejaculated or non-ejaculated sperm

Gwendolyn H. Woldringh

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Voor mijn vader

Contents

Chapter 1

Introduction

Introduction

In the late 1960s, Professor Edwards and Doctor Steptoe started collaboration and developed In Vitro Fertilisation (IVF). They made the breakthroughs in IVF that have revolutionised the lives of infertile couples worldwide, that started with the birth of the first IVF-baby Louise Brown on July 25, 1978.[1] For the development of IVF, Professor Edwards won the Nobel Prize in 2010.

The prevalence of infertility worldwide is estimated to be 9% and approximately 50% demand for medical services.[2] Male factors account for approximately half of the cases.[3] However, IVF could not help the infertile couples with severe male factor. The introduction of intracytoplasmic sperm injection (ICSI) in 1992 enabled the management of severe male factor infertility with high success rates.[4] ICSI involves the injection of a preselected spermatozoon into a mature oocyte after ovarian hyperstimulation and oocyte retrieval. For men with azoospermia, ICSI with sperm retrieved at the level of the epididymis or testis is the sole possibility to father their own genetic progeny.

The use of IVF has increased, and its effectiveness has grown over time, especially with the introduction of embryo cryopreservation[5] and ICSI. To date, in Europe more than 350.000 IVF/ICSI cycles are started annually, corresponding to 850 cycles per million inhabitants.[6] The proportion of ICSI versus standard IVF procedures increased from 39.6% in 1997 to 66.5% in 2006.[7] A similar trend has been observed in the USA with a percentage from 11.0% in 1995 to 57.5% in 2004.[8] There is a more liberal use of ICSI in couples with unexplained infertility, light to moderate impairments of semen quality and in patients with various mixed causes of infertility or few oocytes. So in many countries there may be an overuse of ICSI, compared to IVF, without good medical evidence that it is beneficial neither for the patients or society.[7]

In 2006 the clinical pregnancy rate per transfer was 32.4% for IVF and 33.0% for ICSI. It can be estimated that 1.0% to 4.1% of the overall number of live births are the result of IVF or ICSI.[6] These numbers indicate that artificial reproductive techniques (ART), and especially ICSI, are available and practiced successfully on a large scale. Along with the success story, less attention is paid to safety and the potential risks and complications of this treatment.[3,9]

Risks and complications

In ICSI the risks are theoretically increased for several reasons.[10] Firstly, the risks of the female gamete: injection of the oocyte might cause damage to the ooplasm or meiotic spindle apparatus; foreign substances or contaminants might be injected in the oocyte; and moreover an anomalous female gamete, that otherwise would be bypassed by natural selection, might be fertilized. Secondly, the risks of the male gamete: sperm carrying DNA anomalies, i.e. breaks and aneuploidy,[11,12] Y-chromosome deletions,[13] or structural defects, might be injected. When sperm is surgically retrieved from the epididymis or from the testis, there are additional risks, like ageing after a prolonged stay in the epididymis (in case of obstruction) and incomplete maturation.[14]

Because of all these theoretical risks, there are concerns about the possible adverse effects on birth defects and on the health and development of children, especially when non-ejaculated sperm is used.

Beside possible effect of ICSI treatment for the children, there are possible short-term complications for woman after assisted reproductive technology. The prevalence of these complications is approximately 2%, ovarian hyperstimulation syndrome is responsible for half of all complications.[15] Furthermore, the high incidence of multiple pregnancies increases the complication risks, but also singleton pregnancies have a higher complication risk such as pre-eclampsia.[16,17]

ICSI with non-ejaculated sperm

Azoospermia is present in approximately 5% of all investigated infertile couples[3] and is found in 10% of male infertility cases.[18] Azoospermia can be divided into two groups: obstructive azoospermia (OA) with a normal spermatogenesis and non-obstructive azoospermia (NOA) as the result of a testicular failure. Since the introduction of ICSI, it is possible for these couples to father their own progeny by using sperm retrieved by percutaneous epididymal sperm aspiration (PESA), microsurgical epididymal sperm aspiration (MESA) or testicular sperm extraction (TESE). PESA and MESA are eligible in cases of obstructive azoospermia (OA) with an assumed normal spermatogenesis. In cases of non-obstructive azoospermia (NOA), as the result of a testicular failure or in cases of unsuccessful PESA or MESA, TESE is indicated.

From 1994, ICSI in the Netherlands was carried out either with ejaculated semen or with non-ejaculated semen. However, a few years later concerns arose in the Dutch society about the health of the children born after ICSI with non-ejaculated sperm, referring to unknown risks of using aged (epididymal) or immature (testicular) sperm cells. This led to a national moratorium for the application of ICSI with non-ejaculated sperm in 1996. With support from the Dutch government, studies were set up to obtain more information about these risks. Preclinical studies showed that motile epididymal sperm cells did not show increased DNA abnormalities as measured by TUNEL and CMA3 assays.[19,20] Furthermore, studies in other countries showed no increase in the number of congenital defects in children born after ICSI in combination with MESA, PESA and TESE compared to ICSI with ejaculated sperm.[21,22,23] Therefore, the Dutch government agreed in 2001 with the start of a new prospective and multicentre clinical study to confirm whether sperm retrieved by PESA or MESA in case of an obstructive azoospermia can be used safely for ICSI. From June 2007, ICSI with sperm retrieved by TESE followed, on the same condition.

ICSI children

Worldwide there are around 2,5 million children conceived after an ICSI treatment (ICMART, personal communication). The proportion from non-ejaculated sperm is not known, but it should be quite low, certainly less than 5 % worldwide. The proportion of ICSI versus standard IVF procedures and the clinical pregnancy rate per transfer is still growing, so the amount of ICSI children will also grow in the coming years. Together with this ongoing progress, it is important to follow these children on karyotyping, congenital malformations, health, growth, development and fertility. These items and the knowledge of an increased risk of Y-chromosome deletions and of cystic fibrosis, are important for counselling the future parents. Y-chromosome deletions, mainly in the AZFc region, are found in approximately 10% of azoospermic men and 6% of men with severe oligozoospermia.[24] This Y-chromosome deletion will be transmitted to the male offspring. One of the causes of azoospermia is congenital bilateral absence of the vas deferens (CBAVD), these men carry mutations in the cystic fibrosis transmembrane regulator (CFTR) gene.[25] Their offspring has an increased risk of cystic fibrosis, so genetic testing of the partner and counselling of the couple is recommended.

A significantly higher rate of de novo chromosomal anomalies such as sex chromosome aneuploidies and structural chromosome anomalies, notably reciprocal translocations, has been observed in ICSI-mediated offspring.[26] This has led to a situation in which we do not know to what extent ART procedures increase the genetic load and how this could be reflected in the expressed congenital malformations.

Large follow-up studies have revealed a small though statistically significant increase in the incidence of certain birth defects in IVF and ICSI children compared to naturally conceived infants.[27,28] This increase of birth defects in singletons born after ART might be related with the hormonal treatment for infertility or the procedure itself (oocyte retrieval, culture medium, embryo transfer), but the underlying cause of infertility or its determinants might play a role as well.[29] Another effect may be a lower birth weight of the IVF and ICSI singletons than children conceived spontaneously, even after correction for gestational age, gender and maternal factors.[30,31] In addition increased rates of small-for-gestational age (SGA) have been reported for IVF and ICSI singleton pregnancies.[32,33] The mechanism behind this difference in birth weight is unknown, but beside the explanations mentioned before, the pre-existing condition of the mother or double-embryo transfer might play a role.[34,35] Whether the effect is restricted to birth weight or reflects a general delay in growth or influences the health and/or development of the children is not yet known.

Although in the literature increased rates were found of abnormal karyotypes and major malformations of the ICSI-children, it should be considered that most study groups were small and heterogenic with numerous potential biases. Standardized methodology for follow-up studies after ART are necessary with well-defined groups, such as ICSI with ejaculated sperm, ICSI with epididymal sperm and ICSI with testicular sperm.

Aim and outline of the thesis

The main aim of the thesis was to follow the children conceived after ICSI with ejaculated and especially with non-ejaculated sperm. A questionnaire was developed with questions about parental, pregnancy and child factors, including gestational age, pregnancy complications, mode of delivery, weight at birth and several measure points in the first four years, presence or absence of malformations and neonatal problems.

These questionnaires were sent to the parents of IVF and ICSI children at one year and four years of age. All children were born after a treatment in the Radboud University Nijmegen Medical Centre. The same questionnaires were sent to the parents of children born after an ICSI treatment with epididymal sperm, carried out in six centres in The Netherlands. These centres were all dealing with the protocol approved by the Dutch Central Committee on Research involving Human Subjects (CCMO, The Hague, The Netherlands).

This thesis is divided in two parts, with an intermezzo between. The first part concerns about the follow-up after ICSI with ejaculated sperm with a literature search and analyses of the results of the questionnaires. The intermezzo deals with the first clinical results of ICSI with epididymal sperm in The Netherlands. After these results the study continued with the follow-up after ICSI with non-ejaculated sperm, starting with an explorative study on DNA-arrays, a systematic review about karyotyping, congenital malformations and development of the children and the analyses of the results of the questionnaires of the study groups. In detail, the main questions of this thesis are:

- **Follow-up after ICSI with ejaculated sperm**

    1. What are the theoretical risks and complications of ICSI for women, men and children? (**Chapter 2**)

    2. Is there an association of decreased ovarian reserve in woman with pre-eclampsia? (**Chapter 3**)

    3. What is the weight at birth and the longitudinal growth in the early childhood of IVF and ICSI children? (**Chapter 4**)

- **Intermezzo**

    4. What are the first clinical results of ICSI with epididymal sperm after restarting this treatment under conditions in the Netherlands? (**Chapter 5 and 6**)

- **Follow-up after ICSI with non-ejaculated sperm**

5. Are there differences between the incidence of de novo genomic copy number changes in a group of ICSI children and a control group of naturally conceived children? (**Chapter 7**)

6. What is already published about karyotypes of fetuses, congenital anomalies and follow-up of the children born after ICSI with epididymal or testicular sperm? (**Chapter 8**)

7. Are there differences in the results of the follow-up of children born after ICSI with epididymal sperm compared with children conceived after IVF and ICSI with ejaculated sperm? Additionally, are there differences at two years of age in development of children born after ICSI with epididymal sperm in comparison with the Dutch reference group? (**Chapter 9**)

8. What are the overall results and conclusions of all presented studies? A general discussion. (**Chapter 10**)

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Part 1

Follow-up after ICSI with ejaculated sperm

Chapter 2

Risks and complications of ICSI

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Introduction

The incidence of couples seeking fertility treatment in the western world is estimated to be 15%,[1] and male factors account for about half of the cases.[2] Since the birth of Louise Brown in 1978, in vitro fertilization (IVF) has become a widely used treatment for infertile couples. The introduction of intracytoplasmic sperm injection (ICSI) in 1992 revolutionized the management of severe male-factor infertility, with high success rates.[3] ICSI involves injecting a preselected spermatozoon into a mature oocyte (Fig. 1) after ovarian superovulation and oocyte retrieval. For men with azoospermia, ICSI with sperm retrieved at the level of the epididymis or testis is the sole possibility to father their own genetic progeny.

In Europe, >250 000 IVF/ICSI cycles are started annually, which accounts for 500–1500 cycles per million inhabitants per year.[4] The numbers of reported cycles continued to increase in the last few years, with an increase of 37% from 1997 to 2000.

The mean (range) proportion of ICSI in different countries is 44 (24–68)%. In the European countries in 2000, the clinical pregnancy rate per transfer was 28.4% for IVF and 28.7% for ICSI. It can be estimated that ≈3% of the overall number of live births are the result of IVF or ICSI.[5] These figures indicate that artificial reproductive techniques (ARTs) are

Figure 1 Injection of a preselected spermatozoon in to a mature oocyte.

practiced successfully on a large scale. Along with the success story, less attention is paid to safety and the potential risks for the offspring, and to the consequences of the high rate of multiple pregnancies.[6]

Theoretical risks of ICSI

Natural conception is associated with failures in all stages, ranging from fertilization through birth defects to developmental abnormalities and infertility in the offspring. In ICSI the risks are theoretically increased for several reasons.[7] Firstly, there are risks to the female gamete: injection of the oocyte might cause damage to the ooplasm or meiotic spindle apparatus; foreign substances or contaminants might be injected in the oocyte; and moreover an anomalous female gamete, that otherwise would be bypassed by natural selection, might be fertilized. Secondly, there are risks to the male gamete: sperm carrying DNA anomalies, i.e. breaks and aneuploidy,[8,9] Y-chromosome deletions,[10] or structural defects, might be injected. Most infertile men with congenital bilateral absence of the vas deferens (CBAVD) carry mutations in the cystic fibrosis transmembrane regulator (CFTR) gene;[11] their offspring have an increased risk of cystic fibrosis, so genetic testing of the partner and counseling of the couple is recommended. When sperm is surgically retrieved from the epididymis or from the testis, there are additional risks, e.g. incomplete maturation and ageing after a prolonged stay in the epididymis (in case of obstruction).[12]

Finally, the process of genomic imprinting might be incompletely installed during gametogenesis, or maintained during embryonic development.[13] Mouse developmental genetic research and the genetics of some rare developmental disturbances in humans, such as Angelman syndrome, Prader Willy and Beckwith-Wiedemann syndrome, clearly show that some chromosome regions derived from oocytes rather than sperm are different for a subset of ≈50 genes. Depending on the gene, during prenatal life or into adulthood, there is monoallelic expression of either the male-derived allele or the female-derived allele. When mouse zygotes are reconstructed to contain genetic material from the father only, the balance during early embryogenesis between the embryo proper and the embryonic part of the placenta shifts towards the latter. These observations agree with the developing insight that genomic imprinting is specifically important for placental functioning. Similarly, during mouse in vitro development before implantation, the pattern of imprinting can be upset by the type of medium used and most easily for the placenta.[14]

Of most human imprinting defects that are associated with ART, involving IVF and ICSI, there was hypomethylation on the genomic areas concerned for the maternal allele.

Together with observations in farm animals, this led to the suspicion that in vitro embryo culture conditions are responsible. Consequently, imprinting disorders might result indirectly from ICSI and are probably unrelated to sperm differentiation.

Multiple pregnancies

The major concern of ICSI is the high rate of multiple pregnancies (26%), because two or more embryos are transferred per cycle. The result is that 40% of ICSI-derived offspring are a part of multiple pregnancy.[4] In natural conception a twin is the result of the fertilization of two separate oocytes (1.2% of pregnancies) or a single fertilized oocyte that subsequently divides into two identical structures (0.4% of pregnancies).[15] Although, singleton pregnancies after ICSI have a worse perinatal outcome than unassisted singleton pregnancies, twin pregnancies after ICSI have a better outcome than after natural conception.[16] Perinatal and maternal mortality and morbidity are increased, because of the higher rate of prematurity (<37 weeks of gestation), low birth weights (<2.5 kg) and maternal complications (pre-eclampsia, anaemia, postpartum haemorrhage).

Children born after multiple gestation may suffer long-term consequences of perinatal complications, including cerebral palsy and learning disabilities. Furthermore, parents of multiple births experience more stress, and siblings of multiple births are more likely to have behavioural problems.[17]

Until recently, physicians and patients underestimated the negative consequences of multiple pregnancies.[15] Currently however, elective single embryo transfer (eSET), with the aim of reducing the number of multiple pregnancies while maintaining an acceptable pregnancy rate, is practiced in increasingly many institutions.[18] In the eSET scenario, a multiple pregnancy is regarded as a complication.[19]

Early pregnancy

The percentage of abortion after ICSI is 17.6%, which is similar to the rates after IVF, irrespective of the cause of male infertility and the origin of the sperm.[20] Moreover, it is similar to the overall risk of spontaneous abortion after naturally conceived pregnancies, which is 14–21%. This proportion depends on the woman's age and previous history of spontaneous abortion.[21] However, the percentage of aneuploidy in ICSI conceptus is significantly higher,[22] possibly due to abnormalities in the sperm of patients with ICSI.[8,23] Lower fertilization and implantation rates are reported when testicular sperm of men with unobstructive azoospermia is injected.[24] These data suggest that the theoretical risk in ICSI is partly expressed in reproductive failure in the preclinical stage of pregnancy before implantation, i.e. during fertilization and early embryo development.[25] There was a significantly higher rate of de novo chromosomal anomalies (1.6 vs 0.5% in the normal population, on amniocentesis for a mean maternal age of 33.5 years) in ICSI offspring, relating mainly to more sex chromosomal anomalies and partly to more autosomal structural anomalies.[26] This finding was related to sperm concentration and motility, and not morphology. The significantly higher rate of observed inherited anomalies (1.4 vs 0.3–0.4% in prenatal tests in the general population) was related to a higher rate of constitutional chromosomal anomalies, mainly in the fathers.[27]

Birth defects

After the introduction of ICSI in 1992, several researchers expressed concerns about the possible adverse effects on birth defects, and on the health and development of children, especially when unejaculated sperm is used. Some critics even suggested that ICSI might have a negative impact on the genetic composition of the human race.[28] In the Netherlands this has led to a moratorium on the application of ICSI in azoospermic men using unejaculated sperm. That reasoning appears to be valid from the viewpoint that ICSI bypasses the effective biological mechanisms of sperm selection, and has not been preceded by research. Consequently, the human experience with ICSI is only the experimental record.[29]

In particular, the risk of boys born to couples with male factor subfertility has attracted attention, because for many patients with male factor subfertility, a genetic cause can be suspected.

These include Y-chromosomal microdeletions, X-chromosomal and autosomal aberrations (i.e. Robertsonian translocations), syndromal disorders featuring infertility (i.e. Kallmann's syndrome) and ultrastructural sperm defects with a genetic origin.[30] Theoretically, with ICSI these defects can be transmitted to the next male generation.

Furthermore, there is some evidence that the risk of congenital malformations is increased,[31,32,33] especially after ICSI with surgically retrieved sperm in unobstructive azoospermia.[25,34] The results of these studies are of interest because the chance of chromosomal aberrations and genetic abnormalities is higher in men with unobstructive azoospermia. Unfortunately, too few infants have been investigated to draw valid conclusions.

The major malformation rate (those causing functional impairment or requiring surgical correction) varies in different studies, at 0.7–9.1% for ICSI patients and 0.5–7.2% for naturally conceived children.[35,36] However, the case for a causal relationship between ICSI and adverse effects on the offspring is difficult to make, because in most studies maternal characteristics (e.g. age and parity), comorbidity, life-style (smoking, drinking, drugs), and that ICSI children are more often born prematurely with a low birth weight, are confounding factors. Moreover, the low incidence of malformations demands large-scale studies. In recent large Swedish studies, hypospadias was found more often in a cohort of ICSI male infants.[37] The investigators associated this malformation with paternal subfertility. However, there was also a greater incidence of hypospadias in a cohort of IVF boys, and it was related to maternal progesterone administration.[38]

Imprinting diseases like Beckwith-Wiedemann syndrome and Angelman syndrome are very rare, but for the first syndrome, ART (irrespective of the fertilization technique) increases the relative risk by a factor of ≈6.[39] Large-scale and long-term follow-up studies are necessary to confirm an association between imprinting disorders and ART. Further laboratory research is required to understand the pathogenesis of this association.

Infant development

Since the late 1990s only a few studies have been published on the development of children conceived by ICSI. Most of these studies compared the development of such children and control children during the first 2 years of life, using either the Bayley scales of Infant Development or comparable scales. In 1998, an Australian study[40] found lower scores in the Bayley mental scale for the ICSI offspring at 1 year than for the IVF and natural-conception controls. These differences were statistically significant for boys but not for girls. However, other studies could not corroborate these data. Bonduelle et al.[41] reported a prospective study with a follow-up of 2 years; the overall Bayley mental development scores were similar in the ICSI and IVF offspring and naturally conceived children. Sutcliffe et al.[42] found no difference in neurodevelopment using the Griffiths scales of mental development at up to 15 months old in ICSI-conceived children compared with their naturally conceived peers. A pilot study in Belgium showed that the ICSI-conceived children had a similar psychomotor and intellectual development as the IVF- and naturally conceived children at the age of 5 years.[43]

Risks of ICSI for women

Short-term medical complications of inducing ovulation or retrieving oocytes for IVF are rare. Ovarian hyperstimulation syndrome (1.8%), intraperitoneal bleeding (0.2%), pelvic infections (0.4%) and adnexal torsion (0.13%) have been reported.[44] Another short-term effect of IVF or ICSI treatment is the emotional impact. After an unsuccessful treatment cycle, couples score higher on depression scales and many women have a clinically relevant form of depression.[45]

Hormonal and reproductive factors are involved in the causes of breast cancer and cancers of the female genital tract. Therefore, the long-term effect of fertility drugs on the risk of these cancers has been investigated. Many studies have not been able to reach firm conclusions because of low statistical power, lack of control for important confounders (e.g. causes of subfertility and parity) and short duration of follow-up. In a large-scale cohort study in the Netherlands, after a follow-up of 5–8 years, there was no increased risk of breast and ovarian cancer in women who had undergone IVF compared with subfertile women who had received no IVF.

For endometrial cancer there was a greater risk in those exposed to IVF and in the unexposed group of women with subfertility caused by hormonal disorders.[46] The ESHRE consensus group concluded that there is currently no evidence that ART has any effect on the incidence of genital or breast cancer.[4]

Risks of ICSI for men

Surgical sperm retrieval by percutaneous epididymal sperm aspiration or testicular sperm extraction in case of obstructive or unobstructive azoospermia is a procedure with only minor complications; pain, bleeding, bruising and scarring are the most common.[47] There might be a risk of a decrease in serum testosterone levels after testicular sperm extraction, especially in cases of small testicular volume and hypogonadism, such as patients with nonmosaic Klinefelter syndrome.[48] After an unsuccessful treatment cycle, couples score higher on depression scales.[45] A greater subjective responsibility for the infertility, impact of childlessness on daily life and treatment-related stresses (particularly for sperm aspiration/ extraction methods) is described for men after an ICSI treatment. The men only reported marginally higher depression scores than their controls.[49]

Conclusions

The theoretical risks of ICSI may result in fertilization failure, abortion, birth defects, genetic abnormalities, developmental abnormalities and infertility in the offspring. According to the current state of knowledge, it appears that the incidence of chromosomal abnormalities, including de novo abnormalities, is higher after ICSI than in the general population. This might be a result of the infertility per se rather than the ICSI technique. The incidence of congenital malformations might be slightly higher after ICSI, but more large prospective studies, with naturally conceived children as controls, are needed to address this question definitively.

Special attention is needed for children born after ICSI using epididymal or testicular sperm obtained from men with obstructive or unobstructive azoospermia. Concerns about the use of immature testicular spermatozoa from men with testicular failure require further study. One of the ESHRE consensus recommendations is to offer chromosomal analysis in cases of unobstructive azoospermia and oligozoospermia with $<5 \times 10^6$ sperm/mL, and offer microdeletion testing in men with unobstructive azoospermia and

oligozoospermia of $<1 \times 10^6$ sperm/mL. CFTR gene analysis must be offered in cases of CBAVD. If the genetic abnormality is confirmed, counseling of the couple by a trained genetics specialist must be involved.[4] Another recommendation is to offer midtrimester ultrasonographic screening for congenital malformations, and amniocentesis may be considered.
The conclusion that children conceived by ICSI have similar psychomotor and intellectual development at the age of 5 years as have those conceived by IVF or spontaneously need to be confirmed by multicentre studies.

The increased risk of congenital malformations seems also to be related to preterm and multiple births, so a twin pregnancy is regarded as a complication. The ESHRE consensus meeting agreed that the essential aim of IVF and ICSI is the birth of one healthy child, so elective single-embryo transfer is proposed in a first IVF/ICSI cycle in women aged <36 years if at least one good quality embryo is available.

The question of possible risks of infertility in the offspring, especially boys, is still unanswered, because the oldest children are only 12 years old. It is very difficult to follow these children to their fertile age, because of privacy and the possible stigmatization of these children.

Before starting ICSI treatment it is important to counsel all patients, verbally and by standard patient information material. They must be informed that not everything is known about the health and development of children in their puberty and beyond, and about their fertility.

Finally it is important to have good registries in all countries, including all data on maternal and fetal morbidity and mortality, congenital malformations (with a uniform nomenclature), ART and non-ART procedures.

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Conflict of interest

None declared.

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Chapter 3

Decreased ovarian reserve relates to pre-eclampsia in IVF/ICSI pregnancies

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Abstract

BACKGROUND: Pre-eclampsia affects 2–10% of all pregnancies and is a major cause of maternal and fetal morbidity and mortality. As compared with the general population, IVF pregnancies are associated with a 2.7-fold risk of preeclampsia. An advanced age and associated subfertility in the IVF group reflects a general decrease in ovarian reserve, which itself has been linked to cardiovascular disease. We tested the hypothesis that decreased ovarian reserve is associated with pre-eclampsia as a vascular complication in IVF/ICSI pregnancies.

METHODS: In this retrospective case–control study, 41 cases with a history of pre-eclampsia were compared to 82 matched controls without hypertension or (pre)eclampsia. All pregnancies were established after IVF or ICSI. Several indicators of ovarian reserve such as variables related to basal ovarian function and response to hyperstimulation were compared in both the groups by multivariate analysis. The condition of the neonates was evaluated as well.

RESULTS: A higher amount of total administered FSH and FSH per day, together with a lower number of obtained oocytes during IVF treatment, were associated with an increased risk to pre-eclampsia in a subsequent pregnancy. The administered FSH per follicle and per obtained oocyte showed even stronger relationships, the latter having the best predictive value. Neonatal outcome was comparable between the groups.

CONCLUSION: Diminished responsiveness of the ovaries to FSH stimulation in an IVF cycle, reflecting decreased ovarian reserve, is associated with an increased risk of developing pre-eclampsia in a subsequent pregnancy.

Introduction

Pre-eclampsia is a major cause of maternal and fetal morbidity and mortality worldwide. The severity of the disease ranges from a mild disorder with transient hypertension and proteinuria in the later part of pregnancy to a life-threatening disease with seizures and severe fetal distress.[1] Overall, pre-eclampsia affects 2–10% of all pregnancies and accounts for as much as 15–20% of maternal mortality in developed countries.[2] Identifying pregnancies at risk for pre-eclampsia is of great value, because close monitoring is helpful in preventing some adverse events.[3] Numerous studies have tried to identify risk factors or have searched for screening tests to diagnose the disease at an early preclinical stage.[4] However, to date, this pursuit has not resulted in any suitable test for routine use in clinical practice.[5]

As compared with the general population, IVF pregnancies are associated with a 2.7-fold risk of pre-eclampsia[6] and a two-fold increased incidence of small for gestational age.[7] Several reasons have been brought up to explain this phenomenon, such as multiple gestation and advanced age at first ongoing pregnancy.[8] However, these cannot fully clarify the observed association. The advanced age and associated subfertility in this group also reflect a general decrease in ovarian reserve, which itself has been linked with cardiovascular risk as well.[9,10] Consequently, indicators of decreased ovarian reserve, such as elevated basal FSH levels or a diminished response to stimulation by exogenous FSH,[11] may point out those women at risk for development of vascular complications in their subsequent pregnancies.

In this study, we hypothesize that decreased ovarian reserve in women is associated with pre-eclampsia as a vascular complication in pregnancy. To this end, we conducted a retrospective case–control study amongst IVF and ICSI pregnancies. Cases that developed pre-eclampsia were compared to matched controls without hypertension or (pre)eclampsia in pregnancy. Ovarian reserve was assessed by basal levels of FSH and estradiol (E2) and by the response to hyperstimulation with exogenous FSH during IVF/ICSI treatment. These characteristics and subsequent pregnancy outcome variables were compared between the study groups.

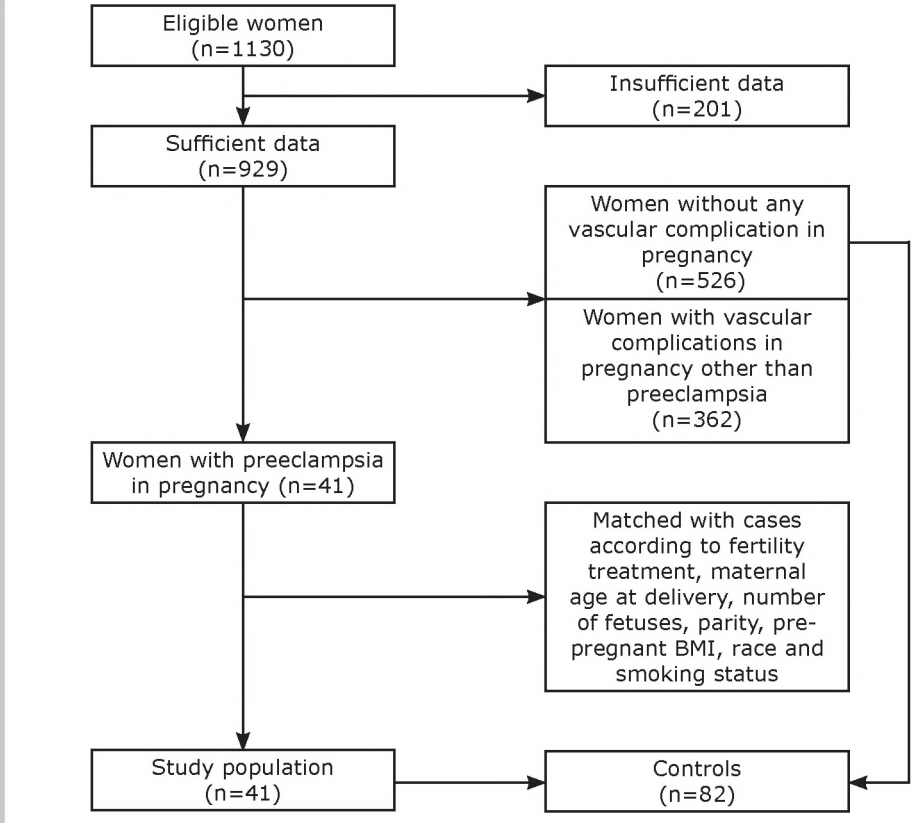
Materials and methods

Patients

In this retrospective case–control study, data were obtained from two databases: (I) objective information on fertility treatment and pregnancy outcome and (II) self-reported data on demographic features and pregnancy complications based on an earlier performed questionnaire study. Verification of reported complications during pregnancy and examination of other data were performed by studying the individual medical record.

A total of 1130 women who were successfully treated by IVF or ICSI in the Radboud University Nijmegen Medical Centre between October 1994 and April 2004 were enrolled in this study. Pregnancies established after transfer of frozen embryos and pregnancies that did not result in a live birth were not included. Of these 1130 eligible women, 201 were excluded because of insufficient data in the database of the questionnaire study (Figure 1). Of the remaining 929, 41 (4.4%) women reported pre-eclampsia during pregnancy, which was confirmed by checking the individual medical records. These women served as cases. For each case, two control women were selected out of a group of 526 women without any reported vascular complication in pregnancy (Figure 1). These 82 controls were matched according to the type of fertility treatment and several characteristics known to influence the risk of pre-eclampsia in pregnancy:[4] the number of fetuses, parity, maternal age at the time of delivery, pre-pregnant BMI (kg/m^2), race and smoking. Smoking was evaluated as three subgroups, referred to 'no smoking', 'smoking but not during pregnancy' and 'smoking also during pregnancy'. In case of more patients matching with the same treatment, the number of fetuses, parity and smoking group, the four patients with the best match for age at the time of delivery were checked for the two best matches for BMI. Because the indication for assisted reproduction treatment (ART) and the duration of subfertility might also have an effect on the development of pre-eclampsia,[12] we studied these variables as well, the latter being defined as the time evolved between the reported date of child wish and the date of ovum pick up minus 14 days.

Figure 1 Determination of case and control groups



IVF and ICSI treatment

Baseline FSH (IU/l) and baseline E2 (pmol/l) were measured on day 3 (±1) of a menstrual cycle before fertility treatment.

All IVF and ICSI cycles were performed according to a standardized ovarian stimulation protocol with pituitary down-regulation with a GnRH-agonist (leuproreline or triptoreline) followed by daily injections with urinary HMG or recombinant FSH (rFSH). At the moment of down-regulation, checked by ultrasound, daily injections with 150 units of FSH were started. Women older than 38 years, those who had a baseline FSH above 10 IU/l or had a poor response (less than four follicles with a diameter >15 mm) in a treatment cycle before, started with a higher daily dose up to 300 units

of FSH. Women known to have polycystic ovary syndrome (PCOS), or with overstimulation in a previous cycle, started with a lower dose of FSH. The total amount of administered FSH was defined as the product of the dose of administered FSH per day and the total number of days of administration. An injection of 10000 units of hCG was given 36 h before oocyte retrieval. The luteal phase was supported by injections of hCG or vaginally administered progesterone. The maximal achieved E2 concentration represented the highest level of E2 measured during FSH stimulation in the cycle leading to conception and was determined by measuring the serum E2 concentration on the day of hCG-injection, 2 days before oocyte retrieval. Maximal endometrial thickness (mm) was the greatest thickness of the triple-layer intracavitary-lined endometrium reached during stimulation. The number of follicles with a diameter >9 mm was determined on the day of hCG injection. The fertility laboratory determined the number of obtained oocytes and the number of embryos usable for transfer. An embryo usable for transfer was defined as an embryo with two pronuclei on day 1 and which had reached the 2- to 8-cell stage on day 3.

From these data, the administered FSH units per follicle and per obtained oocyte were calculated, defined as the total amount of administered FSH divided by the number of follicles and the number of obtained oocytes, respectively. The number of the treatment cycles in which the women conceived was examined as well.

Pregnancy course and outcome

Pre-eclampsia was defined according to the criteria of the Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy,[2] which included gestational hypertension (repeated blood pressure measurements of >140 mm Hg systolic or >90 mm Hg diastolic) and proteinuria (urine protein creatinine ratio of ≥0.3g/10 mmol or dipstick test ≥1+ for protein) after 20 weeks of gestation. Gestational age at delivery was defined as the difference between the date of oocyte retrieval and the date of delivery with 14 days added. Birthweight was measured, and for determination of the birthweight centile, we used the Dutch national reference curve, which adjusts for parity, gestation and sex of the infant.[13] Apgar score and sex of the children were evaluated as well.

Statistics

Statistical analysis was carried out using the Statistics Package for Social Sciences (SPSS) 12.0.1 for Windows. Variables, detailed as median and range or as percentage, were evaluated non-parametrically by using Mann–Whitney U-test and Fisher's exact test, where appropriate. We considered a difference statistically significant when $P < 0.05$. If applicable, correlations were evaluated by Spearman's Rho correlation analysis. To identify actual independent factors, we performed a multivariate backward stepwise logistic regression analysis that included as covariates those variables that were found to be correlated with the study variables. Receiver operation characteristic (ROC) curves were constructed from the possible predictors to explore their predictive values. We calculated the Mantel–Haenszel common odds ratios and 95% CI at the threshold points that yielded to a reasonable value in sensitivity and specificity.

Results

The demographic characteristics of the women with preeclampsia and the control group are summarized in Table I. Both the subgroups were comparable with respect to duration of subfertility, indication for IVF or ICSI and all matching criteria, such as maternal age at delivery, pre-pregnant BMI, maternal smoking status, parity, number of fetuses and type of fertility treatment. There were no pregnancies with more than two fetuses in the study population, and in case of multiparity, it was always a pregnancy of the second child of the woman. All women were Caucasians. In the both groups, the median treatment number of the conception cycle was 2 [range: 1–6].

Leuproreline versus triptoreline, urinary HMG versus rFSH and the used medication during the luteal phase were comparable between the group of women with pre-eclampsia and the control group.

Table I Demographic characteristics of the study groups

	Pre-eclampsia (n = 41)	Controls (n = 82)	P-value
Maternal age at delivery (years)	33.6 [26.3-40.9]	33.6 [26.6-39.8]	0.760
Pre-pregnant body mass index (kg/m^2)	22.9 [18.8-31.6]	22.6 [17.8-32.8]	0.408
Maternal smoking			
No smoking (%)	75.6	81.7	0.479
Smoking but not during pregnancy (%)	9.8	7.3	0.730
Smoking also during pregnancy (%)	14.6	11.0	0.569
Nulliparous (%)	87.8	87.8	1.000
Singleton pregnancies (%)	53.7	53.7	1.000
Assisted reproductive technologies			
IVF (%)	41.5	41.5	1.000
ICSI (%)	58.5	58.5	1.000
Duration of subfertility (years)	4.2 [1.2-11.7]	3.9 [0.7-14.3]	0.953
Indication for assisted reproductive technologies			
Tubal factor (%)	7.3	9.8	0.750
Endometriosis (%)	9.8	9.8	1.000
Male subfertility (%)	58.5	61.0	0.846
Unexplained subfertility (%)	19.5	17.1	0.805
Cervical hostility (%)	4.9	2.4	0.600

Data are expressed as median [range] or percentage.
P-value is calculated non-parametrically (Mann-Whitney U-test or Fisher's Exact Test).

Data of the baseline, stimulation and response variables are summarized in Table II. There was no difference in baseline FSH and E2 levels between the two groups. As compared with the controls, more pre-eclamptic women received an increased daily dose of FSH (Figure 2), although the median in both the groups was the same. There were 19 (47.5%) women in the pre-eclamptic group, who received >150 units of FSH daily as compared with 22 (26.8%) women in the control group (P = 0.027). The first group also received a higher total dose of FSH. A lower number of oocytes was obtained in this group. After assessment of multivariate backward stepwise logistic regression analysis on the baseline, stimulation and response

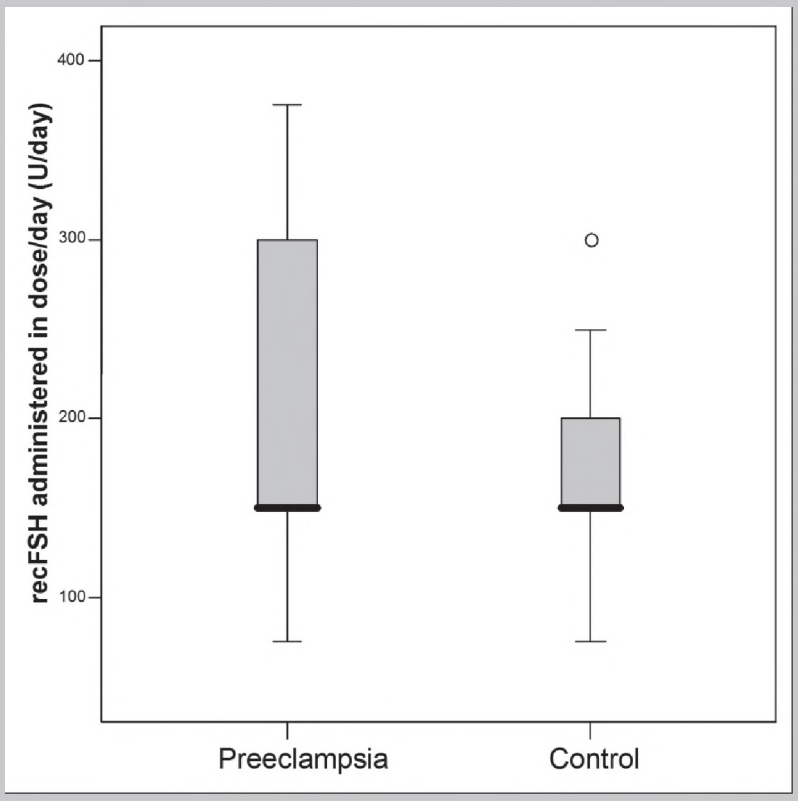
variables, two independent variables—the total dose of administered FSH and the number of obtained oocytes—remained. Consequently, the calculated administered FSH per obtained oocyte was higher in the pre-eclamptic group as compared with the controls. Considering the effect of age on the incidence of pre-eclampsia, as well as an observed correlation between age and dose of administered FSH, we performed a multivariate analysis including maternal age as covariate as well. Moreover, another multivariate analysis was carried out to examine the possible influence of the used FSH and GnRH-agonists and the medication for luteal support. The results showed that maternal age, as well as the medication used in cycle control and for support in the luteal phase, had not influenced the results (data not shown).

Table II Baseline, stimulation and response variables of the study groups

	Pre-eclampsia (n = 41)	Controls (n = 82)	P-value
Baseline			
FSH (IU/l)	5.7 [2.9-11.7]	6.3 [2.8-13.0]	0.304
Estradiol (pmol/l)	110 [66-250]	135 [38-240]	0.462
Stimulation			
Administered FSH in dose/day (U/d)	150 [75-375]	150 [75-300]	0.033
Days of administration	11 [8-15]	10 [7-16]	0.381
Total administered FSH (U)	1800 [900-4125]	1650 [750-4200]	0.027
Response			
Maximal estradiol (pmol/l)	7200 [440-15000]	7000 [1100-14000]	0.931
Maximal endometrial thickness (mm)	11.5 [5.0-18.2]	12.0 [7.3-18.5]	0.329
Number of follicles (>9mm)	12 [4-33]	14 [4-28]	0.069
Number of obtained oocytes	8 [2-20]	11 [4-24]	0.029
Number of usable embryo's	5 [2-17]	6 [1-18]	0.101
Indexes			
Administered FSH/follicle (U)	159 [38-660]	128 [45-500]	0.016
Administered FSH/obtained oocyte (U)	240 [50-1300]	163 [63-540]	0.004

Data are expressed as median [range].
P-value is calculated non-parametrically (Mann-Whitney U-test).

Figure 2 Box plot of administered FSH in dose per day



With respect to responsiveness, there were no differences in the maximally reached E2 levels, endometrial thickness, number of follicles with a diameter >9 mm and usable embryos. Yet, in line with our findings in FSH per obtained oocyte, the FSH administered per follicle was also higher in the pre-eclamptic subgroup as compared with controls.

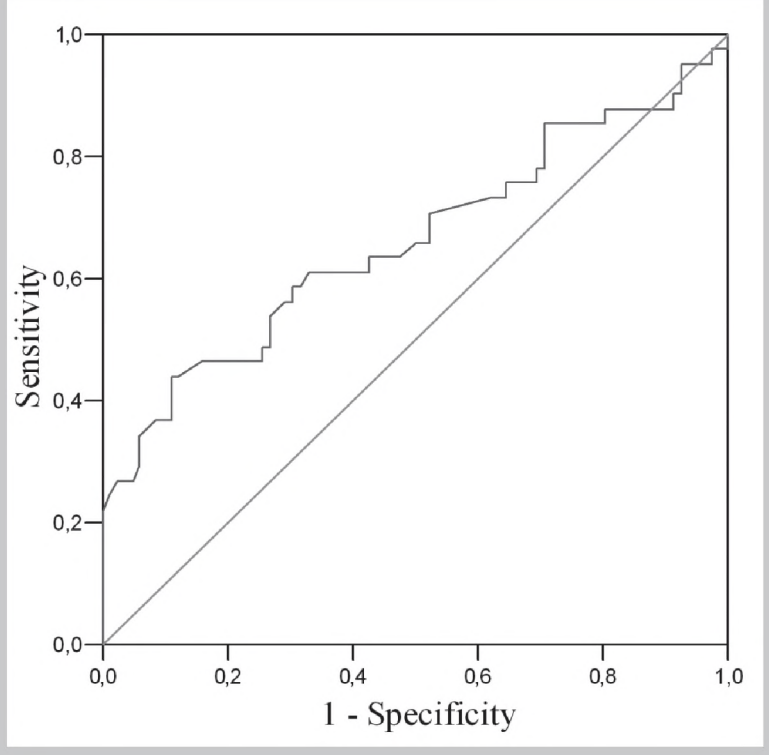
The results of the univariate analysis of predictive variables at given threshold points as assessed after ROC construction are summarized in Table III. At these threshold points, the administered FSH per obtained oocyte had the best, yet the modest, predictive power. The ROC curve of this variable is shown in Figure 3.

Table III Univariate analysis of variables associated with the occurrence of pre-eclampsia

	Sensitivity	Specificity	AUC	OR	95%CI
Administered FSH in dose/day (>175 U/d)	46.2	72.8	0.610	2.355	1.075-5.161
Total administered FSH (>1550 U)	73.2	47.6	0.622	2.474	1.094-5.591
Number of obtained oocytes (<10)	62.2	58.5	0.620	2.323	1.081-4.991
Administered FSH/follicle (>154 U)	53.7	70.7	0.633	2.798	1.287-6.083
Administered FSH/obtained oocyte (>196 U)	61.0	67.1	0.659	3.183	1.461-6.932

AUC = area under the curve, OR = Mantel-Haenszel common odds ratio, CI = confidential interval.

Figure 3 Receiver operation characteristic (ROC) curve of administered FSH per obtained oocyte related to the development of preeclampsia



The pregnancy outcomes of the study groups are summarized in Table IV. For singleton as well as for twin pregnancies, the gestational age at delivery and birthweight of all children were lower in the pre-eclamptic subgroup. Between the two subgroups, there were no differences in observed sex, birthweight centile and umbilical arterial pH at birth and Apgar score after 5 min.

Table IV Pregnancy outcome of the study groups

	Pre-eclampsia (n = 41)	Controls (n = 82)	P-value
Singleton pregnancies			
Gestation at delivery (weeks)	37.9 [30.6-40.6]	40.3 [36.7-42.3]	< 0.001
Birth weight (grams)	2935 [1130-4250]	3265 [2465-4680]	0.018
Birth weight centile	54 [8-99]	52.5 [1-99]	0.703
Umbilical arterial pH at birth	7.28 [7.13-7.36]	7.25 [7.00-7.38]	0.667
Apgar score 5 minutes	9 [4-10]	10 [7-10]	0.318
Twin pregnancies			
Gestation at delivery (weeks)	35.7 [31.0-39.6]	38.0 [33.0-40.4]	0.001
Birth weight (grams)			
First child	2280 [1392-2990]	2762.5 [2005-3525]	0.001
Second child	2245 [1138-3270]	2722.5 [1800-3660]	0.003
Birth weight centile			
First child	22 [7-93]	41.5 [1-95]	0.360
Second child	22 [2-72]	31.5 [1-88]	0.186
Umbilical arterial pH at birth			
First child	7.26 [7.20-7.39]	7.26 [7.04-7.39]	0.642
Second child	7.25 [7.13-7.34]	7.25 [7.04-7.33]	0.330
Apgar score 5 minutes			
First child	10 [8-10]	10 [6-10]	0.885
Second child	9 [4-10]	9 [4-10]	0.670

Data are expressed as median [range] or percentage.
P-value is calculated non-parametrically (Mann-Whitney U-test or Fisher's Exact Test).

Discussion

Pre-eclampsia affects 2–10% of all pregnancies and relates to substantial maternal and fetal morbidity and mortality.[14] Women conceiving through treatment by ART seem to more likely develop pre-eclampsia.[6,15] In our study, we explored a possible association between ovarian reserves, as measured by basal ovarian function and response capacity to FSH stimulation on one hand and the development of pre-eclampsia as pregnancy-related vascular complication on the other. We observed that a decreased responsiveness of the ovaries to FSH stimulation, as indicated by the need for a higher amount of total administered FSH and FSH per day and a lower number of obtained oocytes during an IVF or ICSI cycle, was associated with an increased risk to develop pre-eclampsia in a subsequent pregnancy. The administered FSH per follicle and FSH per obtained oocyte showed an even stronger relation, with the latter being the most powerful predictor of all variables studied.

Patients with PCOS have a higher risk of pre-eclampsia.[16] Because PCOS patients generally have a high ovarian response to FSH,[17] these women may be overrepresented in the group with low FSH dosages per obtained oocyte. This may have biased our results. However, the number of patients with PCOS in our group was too small to draw conclusions on this possible bias.

In our study, basal ovarian function, as assessed by baseline FSH and E2 concentrations, did not relate to the development of pre-eclampsia. Although basal FSH is a frequently used marker for ovarian reserve, its value in clinical practice is argued.[18] The response of the ovaries to hyperstimulation might be superior in reflecting ovarian reserve as compared with basal functions.[19] This seems to be in line with our findings with respect to the relation with pre-eclampsia.

To our knowledge, this is the first study examining a relation between ovarian reserve and vascular complications in pregnancy. Although we found no similar studies to compare our results with, several authors have examined the response to hyperstimulation in ART in relation with treatment success. An association between diminished responsiveness of the ovaries and decreased pregnancy rates has been found.[20] There is also a higher chance of pregnancy loss found as correlation with diminished ovarian reserve.[21] This high loss rate was likely to be because

of fetal aneuploidy, which was higher among women with elevated FSH concentrations,[22] but there are also other possible factors affecting the chance of an ongoing pregnancy in patients with diminished ovarian reserve.

Pregnancies after oocyte donation are associated with an increased risk of pregnancy-induced hypertension compared with pregnancies after IVF with own oocytes.[23] On the one hand, this may be viewed upon as an immunological imbalance between 'graft' and 'host'. On the other hand, the ovarian failure may also indicate a generalized ageing of the system, including vascular system, ultimately leading to the vascular disease of pre-eclampsia. This latter explanation is supported by the observation that patients with primary ovarian failure are at a higher risk for preeclampsia after oocyte donation.[24]

An association between decreased ovarian reserve and an increased risk of pre-eclampsia may have implications for several reserve like those with a family history of a premature menopause, or having a history of chemotherapy, radiotherapy, pelvic surgery or infection,[25] might be counselled for closer monitoring during pregnancy. Women without these risk factors but showing a low response to hyperstimulation could also be considered to be at increased risk for developing pre-eclampsia if pregnancy should occur. Unfortunately, at the chosen threshold point in the ROC curve, our most powerful variable showed only a modest discriminative power, which makes it hardly suitable for routine use in clinical practice. More research on these and other potential predictors is needed.

Because maternal vascular complications in pregnancy relate to neonatal morbidity as well,[1] we studied several variables associated with the condition of the newborn. Apgar score and umbilical arterial pH at birth did not differ between both the study groups. In accordance to other studies, we observed a shorter gestation at delivery in the preeclamptic subgroup, which was expected, considering the tendency to induce labor artificially in case of pre-eclampsia. Although these children had a lower birthweight, after adjustment for gestational age, parity and sex, the outcome between both the groups were comparable. This is in contrast with the findings of several other studies that state that birthweight even after adjustment for gestational age is lower in children born to mothers with pre-eclampsia.[26] Possibly we did not observe this in our study because

of the limited number of cases.

The selection of cases in this study was based on reported complications during pregnancy, and although these were all verified in both cases and controls, the reported incidence of pre-eclampsia was low as compared with that of the general population, especially because pre-eclampsia is stated to occur more frequently in pregnancies resulting from ART.[6] Probably there were women who were not aware of their clinical condition before delivery, in that case we did not select them through their self-report. In addition, the low BMI amongst our studied population may also have affected the incidence of pre-eclampsia. This figure may have been biased by our local selection protocol before IVF, which excludes women when having a BMI above 32 kg/m^2, because obesity is associated with lower changes for live birth after IVF and ICSI,[27] increased rates of miscarriage, gestational diabetes, hypertension and mechanical problems during delivery.[28] Despite the considerable number of women who initially enrolled in this study, the low number reporting pre-eclampsia resulted in a small case group that led to a modest power. Considering this, and because the used case–control design has the tendency to overestimate found relations, a larger prospective study is needed to verify our results.

In conclusion, diminished responsiveness of the ovaries to FSH stimulation in an IVF cycle, which reflects a decreased ovarian reserve, is associated with an increased risk of developing pre-eclampsia in a subsequent pregnancy. We speculate that reduced ovarian responsiveness also reflects diminished vascular reserve capacity, the latter giving rise to pregnancy-associated vascular complications.

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Chapter 4

Weight of in vitro fertilization and intracytoplasmic sperm injection singletons in early childhood

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Abstract

Birth weight and longitudinal growth in the first 4 years of life of term singletons conceived with the use of IVF and intracytoplasmic sperm injection (ICSI) were compared with those of naturally conceived singletons. Although IVF and ICSI singletons had a statistically significantly lower birth weight than naturally conceived singletons, the average individual weight curves showed that this difference was lost before the age of 4 years in all subgroups: IVF, ICSI, boys, and girls.

Singletons conceived through IVF and intracytoplasmic sperm injection (ICSI) have a lower birth weight than children conceived spontaneously, even after correction for gestational age, sex, and maternal factors.[1] Whether the effect is limited to birth weight or reflects a general delay in growth is not yet known. Reduced birth weight and delayed growth are not without health consequences.[2] Epidemiologic studies have established associations between intrauterine and extrauterine growth restriction and the risk of development of health problems in later life, for example, cardiovascular disease.[3] Only a few studies have investigated postnatal growth of IVF children and ICSI children as,[4,5] but a longitudinal growth pattern with at least four measurements of the children has never been published before.

The objective of our study was to compare birth weight and longitudinal growth in the first 4 years of life of IVF and ICSI term singletons with those of term singletons of a national reference group. In addition we investigated whether there are differences within these groups for treatment (IVF, ICSI) and sex (male, female).

The study included 347 IVF and 330 ICSI (with ejaculated sperm) term singletons (gestational age of ≥37 weeks) conceived at the Radboud University Nijmegen Medical Center and born between June 1995 and December 2003. Information on fertility treatment and pregnancy outcome was retrieved from medical records. In addition, questionnaires were sent to the parents of all children at the first and fourth birthday to obtain data on parental, pregnancy, and child factors. These questionnaires included questions about weight at 1, 3, 4, 12, and 18 months and 2, 3, and 4 years of age. These points of measurement are part of the protocol of the regular periodic health examinations by general practitioners at municipal infant welfare centers in the Netherlands. The weight data are documented on a card that the parents take home. The parents were asked to use these weight data to fill in the questionnaire.

The reference group included a total of 5,059 term singletons, from a large population-based cross-sectional growth study in the Netherlands of children between 0 and 5 years measured in 1996 and 1997.[6] Measurements of weight after birth were performed by a general practitioner during the regular periodic health examinations at the municipal infant welfare centers, at the same intervals as in our study population. Statistically significant differences

($P < 0.01$ using the Fisher's exact test) between the IVF and ICSI groups and references were seen in smoking behavior during pregnancy and in birth order. More IVF and ICSI mothers did not smoke during pregnancy (IVF 89.3%, ICSI 88.2%, and the references 76.7%). Moreover, there were more firstborns in the IVF and ICSI groups (respectively 67% and 77.0%) compared with the reference group (45.1%).

A linear regression model was developed to estimate the average weight curves in the two sex categories of the reference group, separately. The dependent variable was the logarithmic transformed weight. The independent continuous variable was the logarithmic transformed age (days after birth).

The mean birth weights were calculated from the observed data, and the weights from 1 month to 4 years were calculated by using the linear mixed model. We found that the differences between the IVF and ICSI group were never statistically significant, and therefore the results of the IVF and ICSI combined groups also are presented.

Table I shows the mean birth weights and ratios of the different subgroups and their associated references. We found that there was a statistically significant difference (the value 1 is not included into the 95% CI of the mean ratios) between the study groups and their associated reference groups at birth but no longer at the age of 4 years. We also calculated the intersection of the growth curves of the study groups and their associated reference group. Specifically the point of intersection of the study group of IVF boys and the associated reference group occurred before 4 years.

Our study showed that term IVF and ICSI singletons had a significantly lower birth weight than children from a national reference group. This difference in birth weight between IVF and ICSI singletons and reference singletons is in accordance with findings of others.[7] The mechanism behind this difference in birth weight is unknown, but several explanations have been given, such as a late effect of the hormonal ovary stimulation, the IVF-ICSI procedure itself (oocyte retrieval, culture medium, ET), the preexisting condition of the mother, factors related to subfertility, or double ET.[8,9] It was not possible in our study to verify all these explanations. For instance, information on the preexisting condition of the mother or the number of early vanishing twins was lacking. However, because there were no differences between the IVF and ICSI groups, it is not plausible

Table I Weight, ratio to the reference group, and time of intersection with 95% confidence interval at birth and at 4 years by gestational age, sex, and type of conception

			Birth weight		Weight at 4 years		Time of intersection [a]
Gender	Type of conception	n	Mean in grams (95% CI)	Ratio (95% CI)	Mean in grams (95% CI)	Ratio (95% CI)	Year (95% CI)
Boys	IVF	169	3407 (3332-3483)	0.97 (0.95-1.00)	16875 (16576-17179)	1.00 (0.98-1.02)	3.5 (0.1-84.9)
	ICSI	150	3404 (3323-3487)	0.97 (0.95-1.00)	16524 (16184-16870)	0.98 (0.96-1.00)	> 4
	IVF/ICSI	319	3406 (3351-3461)	0.97 (0.96-0.99)	16711 (16486-16939)	0.99 (0.98-1.01)	> 4
	natural	2519	3500 (3480-3520)	1.00 (ref)	16863 (16743-16984)	1.00 (ref)	NA
Girls	IVF	178	3301 (3234-3369)	0.98 (0.96-1.01)	15964 (15644-16291)	0.99 (0.97-1.01)	> 4
	ICSI	180	3264 (3181-3350)	0.97 (0.95-0.99)	15950 (15614-16292)	0.99 (0.97-1.01)	> 4
	IVF-ICSI	358	3282 (3229-3337)	0.98 (0.96-0.99)	15962 (15730-16197)	0.99 (0.97-1.00)	> 4
	natural	2521	3356 (3337-3374)	1.00 (ref)	16184 (16065-16304)	1.00 (ref)	NA

Note: CI = confidence interval; NA = not applicable; Ref = reference group.
[a] Point of crossing of the weight curves of IVF and/or ICSI group and the associated reference group.

that the factors related to subfertility or the ICSI procedure itself are of a great influence. In most cases the indication for ICSI was a male factor and the women had no other fertility problems themselves. Other influences on the birth weight that may play a role are maternal age, parity, and smoking behavior during pregnancy.[10,11] The first two factors could have contributed to the lower birth weight in our study group. Parity was significantly lower in the study group. The age of the mothers of the reference group at time of birth was not available. However, the mean age at delivery in the Netherlands in 1990 was 29.2 years and in 2004 was 31.0 years (http://www.cbs.nl). This is substantially lower than in the study group and is in agreement with previous data.[12] Because significantly more IVF and ICSI mothers did not smoke during pregnancy, the weight difference between IVF and ICSI children and the reference group could have been more pronounced if the smoking behavior had been equal in both groups.[13]

Previous studies reported on postnatal growth of IVF children[14,15] and other studies about ICSI children as well.[4,5] Although almost all studies are reassuring, they have some limitations such as having not differentiated for gestational age, having performed only one single weight measurement at 5 years of age, or having included only IVF children. However, our study differentiated for gestational age, had at least four measurements, and included IVF and ICSI children. Our study shows that the weight difference at birth is lost in both IVF and ICSI children before the age of 4 years.

Ludwig et al.[16] reported in a review an increase in childhood illnesses, in chronic diseases, and in the use of health care in IVF children, which may be related to the lower birth weight and higher frequency of preterm birth. Children with a low birth weight have a risk for development of health problems in later life, for example, cardiovascular disease.[3] For these reasons it is important to know the weight curves of the IVF and ICSI children with a gestational age of<37 weeks as well. In our study, because these groups were too small to describe for a reliable conclusion, we recommend future studies for preterm IVF and ICSI infants with a long follow-up time.

Children with diagnosed growth disorders and those receiving medication known to interfere with growth were excluded from the reference group, but these children were not identified in the study group. It was not possible to exclude the IVF and ICSI children from the reference group, because the type of conception was not known. However, the proportion of IVF children in this reference group will be very low. In 1996, one out of 77 Dutch children was born after an IVF or ICSI treatment.[17] Close to 25% of these children are from a twin pregnancy and 5.8% to 15% of the singletons were born before 37 weeks of gestational age;[18] therefore, the number of IVF and ICSI term singletons in the reference group is <1%.

We compared longitudinal data of IVF and ICSI children with cross-sectional data of a reference group. This approach has disadvantages although it gave us the opportunity to have a large population-based control group. In future studies control groups with longitudinal data are to be preferred, with all confounding factors taken into account. The large sample size (especially the reference group), high response rate, and relatively long follow-up with at least four measurements are the strengths of our study. The scheme with frequent measurements of weight made it possible to assess a detailed longitudinal pattern of growth in both IVF and ICSI children.

We conclude that IVF and ICSI singletons with a gestational age of ≥37 weeks have a significantly lower birth weight than natural conceived singletons. It is reassuring that the longitudinal growth of the IVF and ICSI children is comparable with the growth of natural conceived children and that the difference of birth weight is lost before the age of 4 years.

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Intermezzo

Chapter 5

5

Obstructive azoospermia in men who wish to father children; diagnostics and surgical sperm retrieval

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Abstract

OBJECTIVE: To evaluate diagnostic procedures and surgical sperm retrieval in men with suspected obstructive azoospermia who wish to father children.

DESIGN: Descriptive, retrospective.

METHOD: During the period 1 April 1999-31 December 2001 93 men suspected of having obstructive azoospermia underwent surgical sperm retrieval by means of percutaneous epididymal sperm aspiration (PESA). In each patient a testicular biopsy was performed to determine the Johnsen score (a score ≥ 8 is equivalent to a normal spermatogenesis). Cryopreservation was performed whenever possible. The findings in both percutaneous and surgical sperm retrieval were compared.

RESULTS: In 76 patients (82%) epididymal motile sperm were obtained using PESA. Their Johnsen score on the testis biopsy was 9.1 (range: 7.4-10). In 73 of the patients the Johnsen score was ≥ 8. In the 17 patients (18%) in whom no sperm were found with PESA, the median Johnsen score was 5.8 (range: 2-9.8). Epididymal sperm were not found in patients with a testicular volume < 15 ml. In all 28 patients who had undergone a vasectomy in the past, motile sperm were found along with a Johnsen score ≥ 8. In 23 of the 24 patients with congenital bilateral absence of the vas deferens (CABVD) the Johnsen score was ≥ 8. Cryopreservation was possible in 45 (59%) of all patients and in 5 (35%) of the 13 patients with an unknown cause for the obstructive azoospermia.

CONCLUSION: In men with suspected obstructive azoospermia in whom sperm were found using PESA, a diagnostic testis biopsy provided no additional relevant information about the spermatogenesis. There was always a good spermatogenesis after vasectomy. CBAVD patients probably had at least some focal areas in the testes with normal spermatogenesis. Sperm retrieval and cryopreservation could be carried out less frequently in the case of obstructions with an unknown cause.

Introduction

Until recently, it was not possible for men with azoospermia to have offspring that was genetically their own. The introduction of intracytoplasmatic sperm injection (ICSI) created the option of producing offspring by means of surgically retrieved sperm. On 1 January 2001 the moratorium on ICSI with surgically retrieved (non-ejaculated) sperm was abolished. Treatment of men suffering from histological proved obstructive azoospermia, with epididymally retrieved sperm was permitted, according to the protocol approved by the Central Commission for Human-Centered Research (CCMO).[1]

The prevalence of azoospermia in the Netherlands is an estimated 600 to 800 cases a year. Approximately two thirds of these cases are caused by defects in spermatogenesis, and about one third of the cases are caused either by a functional obstruction or by a mechanical obstruction in the genital tract. The obstructive cases are in men who have azoospermia after vasectomy or (re)vasovasostomy, and men with congenital bilateral absence of the ductus deferens (CBAVD); the latter is diagnosed with 1% to 2% of subfertile men.[2,3] Furthermore, there is a small number of men who have an irreparable obstruction caused by a variety of causes, such as trauma (either or not iatrogenic), infections, a median prostate cyst, or an unknown cause that cannot be found.

So, there are a number of men of whom it is certain that they have an obstruction, for instance those who have had an unsuccessful vasovasostomy. Moreover, there are a number of men in whose cases it is unknown whether or not their azoospermia is obstructive. For the latter, it is important to determine by precise non-invasive diagnostic techniques whether they have either an obstruction or a defect in their spermatogenesis.

In this article we will describe what implications the conditions, mentioned in the protocol, have had for the procedures for the selection of patients and for sperm retrieval. This description will be based on the results of the first 93 men who were examined.

Patients and methods

In a retrospective study the results of percutaneous epididymal sperm aspiration (PESA) and testis biopsies were analysed.

Patients

During the period from 1 April 1999 through 31 December 2001, 93 men with supposed obstructive azoospermia had their sperm retrieved surgically by means of PESA at our hospital. Their average age was 39 years. They all had a testis biopsy taken at the same time for an examination of their spermatogenesis. Before 2001 this was done as part of the diagnostic effort. Whenever possible, cryopreservation of the sperm was carried out for future ICSI treatment.

Obstructive azoospermia became the supposed diagnosis as a result of anamnesis, physical examination and hormone examination (when the follicle-stimulating hormone (FSH) turned out to be below 11 U/l). Semen analysis was carried out at least twice, with a period of at least three months between the two analyses. After all, azoospermia may be temporary, for instance as a result of a severe influenza. In the anamnesis the researchers paid attention primarily to the patients' medical history (such as genital infections, vasectomy, maldescensus testis, orchitis, or torsio testis). During the physical examination, the volume of the testes was examined because it usually corresponds to the number of sperm cells in the ejaculate. Furthermore, it was examined whether the patient had an epididymis and a ductus deferens, and whether the epididymis was stowed. Scrotal sonography was not carried out routinely. All of the CBAVD patients were subjected to a genetic examination (Table I).[4]

Johnsen score

In order to make the diagnosis "obstructive azoospermia" appear more likely, a testis biopt with the size of a rice grain was taken for the determination of the Johnsen score as a measurement of spermatogenesis. The Johnsen score is acquired by giving the tubuli seminiferi a score related to the percentage of either the presence or the absence of the main cell types in spermatogenesis in the order of their maturation.[5] So, the presence of spermatozoa is given the score of 10 to 8; the presence of spermatides (but no further) is given a score of 7.9 to 6; the presence of spermatocytes is given a score of 5.9 to 4; and the presence of only spermatogonia is given a score of 3; a score of 2 is assigned to the

Table I Diagnostics in the case of azoospermia

Anamnesis: case history
Physical examination: testis-volume, presence of ductus deferens, stowage of epididymis
Semen analysis: at least twice
Hormonal examination: value of follicle-stimulating hormone (FSH)
Scrotal sonography
Transrectal prostate sonography: on indication
Genetical examination: in case of congenital bilateral agenesis of the ductus deferens

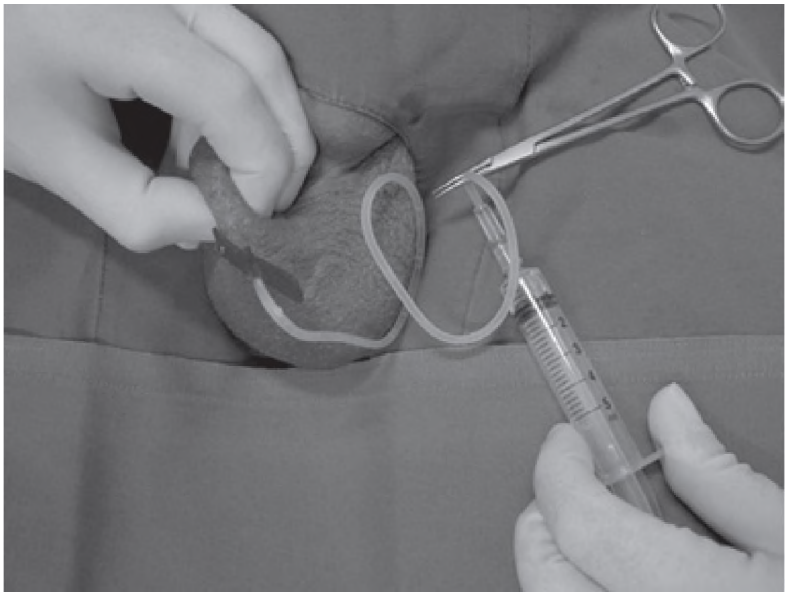
Sertoli-cell-only syndrome, and a score of 1 is given to the absence of stem cells. The definition of normal spermatogenesis is a Johnsen score of 8 or more. The Johnsen score correlates strongly with the quality of spermatogenesis.[5] Essentially, a single one-sided biopt from the largest testis is sufficient. It must be kept in mind that spermatogenesis is not distributed equally between the two testes, and that this distribution is even more unequal in case of a spermatogenetic disorder. Consequently, even with a testis biopsy obstructive azoospermia cannot be diagnosed with certainty.

PESA

At present, in those cases of obstructive azoospermia in which no reconstruction is considered, PESA is the simplest method of sperm retrieval. In case of an obstruction, enough sperm cells are usually found in the epididymis; the percentage of vital (motile) sperm cells usually decreases from proximal (caput) to distal (cauda), depending on the level of obstruction. This is contrary to non-obstructive azoospermia, in which case most motile sperm cells can be found in the distal epididymis.

PESA is carried out as a policlinical operation under local anaesthesia by means of a funiculus block. A (wing-) needle of 17 to 21 gauge is stuck into the epididymis and is withdrawn while sucking in a vacuum (see the figure). The needle is fastened with a small tube to a 5 ml syringe containing 1 ml of NaCl. After that, the small tube is purged into a syringe with a flushing medium (a salt solution to which plasma proteins were added). Its contents are immediately assessed under a microscope by

a laboratory staff member, to assess the presence and the motility of sperm cells. This procedure may be repeated a number of times in each session. In practice, however, it turns out that, if the needle is positioned correctly, the first procedure yields the best output, and that subsequently the output decreases rapidly. The advice is to begin at the side of the largest testis. In case of an insufficient output, the other epididymis may be punctured.



Cryopreservation

In case of a good output, cryopreservation is applied. After concentrating the various aspirations, the sperm cells are mixed with a cryoprotective medium and are frozen in liquid nitrogen in portions of 500 µl each (-196^0 C). If possible, a straw is defrosted immediately after freezing in order to judge the motility of the sperm cell. Thanks to cryopreservation, if there is a good output, the patient in question does not need any sperm aspiration any more during subsequent ICSI attempts. Because cryopreservation can be successfully carried out with only some of the patients, the PESA procedure must be repeated with some patients at the moment of the woman's ovum retrieval. This requires good logistics from the departments of Gynaecology and Urology.

Statistics

For statistical evaluation the X^2 test was used, calculated with the Statistical Package for the Social Sciences (SPSS, version 10.1).

Results

PESA combined with a testis biopsy was tolerated well by all of the 93 patients. Only one patient had a complication. He developed a hydrocele a few weeks after the operation. This was corrected in an operation.

In case of PESA, epididymal motile sperm was extracted from 76 patients (82%). 73 of these patients had a Johnsen score ≥8 and three had a score of 7.4. The median score was 9.1 (the extremes were 7.4 – 10). With 17 patients (18%) no epididymal sperm was found. Their median Johnsen score was 5.8 (extremes: 2 – 9.8). One patient had a Johnsen score of 9.8. In the case of positive sperm retrieval by means of PESA, the average Johnsen score was statistically significantly higher than in those cases when no sperm could be retrieved ($P < 0.001$).

The average volume of the testis from which the biopt was taken, was 20 ml. In case of a testis volume < 15 ml, no epididymal sperm was found ($n = 4$). In Table II the results are presented per cause of obstructive azoospermia. There were no statistical significant differences in FSH value or in testis volume. With all of the 28 patients with a vasectomy and a failed vasovasostomy, epididymal sperm and a Johnsen score ≥ 8 were found. Of the 24 patients with CBAVD, 23 patients had a Johnsen score ≥ 8, and one patient had a score of 7.4; the average Johnsen score in this group was 9.0.

Cryopreservation was successful (meaning that after defrosting, motile sperm cells were found) with 45 (59%) of the epididymal punctures. In advance there were no indications identifying a certain group with whom no cryopreservation would be possible. The group of patients with whom cryopreservation most rarely could be carried out were those who had an unexplained obstruction (5/13; 35%). In this group, epididymal sperm retrieved by means of PESA was found with 13 patients (46%), and the average Johnsen score was 7.2.

Table II Diagnostical results for 93 men with obstructive azoospermia, arranged according to causes of azoospermia

Cause	Number	Mean values			Number (%)	
		FSH (U/l)	testis-volume (ml)	Johnsen score[a]	epididymal sperm[b]	cryo-preservation successful[c]
CBAVD	24	5.7	19	9.0	23 (96)	13 (59)
vasectomie	28	6.5	21	9.0	28 (100)	17 (61)
trauma or injury	13	4.7	20	9.1	13 (100)	10 (77)
unknown	26	6.2	20	7.2	13 (46)	5 (35)
Totally	93	5.8	20	8.5	77 (83)	45 (59)

CBAVD = congenital bilateral agenesis of the ductus deferens
[a] score of testisbiopsy: score of ≥ 8 is equivalent to a normal sprematogenesis
[b] retrieved by percutaneous epididymal sperm aspiration
[c] after defrosting, motile sperm cells were found

Discussion

In this study there was a statistical significant connection between finding epididymal sperm with PESA and a normal spermatogenesis. In case of a supposed obstructive azoospermia, diagnostical PESA that preferably will be combined with cryopreservation suffices, a diagnostical testis biopsy is no longer necessary. Our results are confirmed by other research.[6]

The fact that epididymal sperm is also found with men with a Johnsen score < 8 may be explained by the fact that spermatogenesis is not divided equally among both testes. Locally there may be focal areas with normal spermatogenesis. In such cases a testis biopt may have been taken just beside such an area. The absence of epididymal sperm does not always indicate a spermatogenetic disorder, but may as well be based on an intratesticular obstruction. This was illustrated by one patient without any sperm by PESA with a Johnsen score of 9.8.

The characteristic features of obstructive azoospermia are a normal testis volume and normal FSH values. In a recent American study the FSH values (with a cut-off value of 7.6 U/l) and the size of the testis (with a cut-off value of 4.5 cm) had the highest prognostic value for the diagnosis of obstructive azoospermia.[6] Moreover, inhibin B is mentioned as a good indicator for spermatogenesis.[7] In our study, however, the latter was left aside.

Ultrasound may make a limited contribution to the diagnosis of an obstruction, but it does contribute to tracing defects that are not found during an ordinary physical examination. From two studies in which altogether more than 9000 men participated,[8,9] it appears that a tumour of the testis or a carcinoma in situ is diagnosed with 0.4 – 0.5% of the men. This is 20 to 50 times as often as with fertile men the same age. For this reason it is standard at a number of hospitals to do an ultrasound of the scrotum with each new subfertile man. A transrectal ultrasound of the prostate may also be carried out if there is an indication for doing so. If the semen volume and the pH of the semen are low, the azoospermia may be caused by a distal obstruction such as a median prostate cyst.[10] Such an obstructive median prostate cyst can be visualised by means of transrectal prostate ultrasound. Moreover, the presence or absence of the vesiculae seminales can be ascertained that way.

Genetic examination of patients who have obstructive azoospermia is carried out in case of a CBAVD. This defect can easily be detected by means of a physical examination. Research into mutations in the "cystic fibrosis transmembrane regulator" gene (CFTR) is carried out.[4] Besides cystic fibrosis, this gene may also cause CBAVD. 85% of all men with CBAVD have one or two CFTR-gene mutations, whereas one in every thirty Dutch people is a carrier of one mutation. If the man has a CFTR-gene mutation, a genetical examination of the woman is carried out as well. If the woman also has a CFTR-gene mutation, then, depending on the mutation in question, there is a probability of 25% the child will have cystic fibrosis or a CBAVD. If both the man and the woman are carriers, the couple is sent to a clinical genetic consultant.

In the first surgical sperm retrievals with patients who had an obstructive azoospermia the sperm was retrieved from the epididymis[11,12] or from the testis.[13] In these cases a high percentage of pregnancies was accomplished by means of ICSI. The methods of surgical sperm retrieval included microsurgery at the location of the epididymal ductuli (MESA) or an open testis biopsy with testicular sperm extraction (TESE). However, TESE may have a negative influence on the testis as a result of the formation of fibrosis.[14] Later on, sperm was retrieved by means of less invasive methods, including PESA[15,16] or testicular sperm aspiration (TESA).[17,18,19] The percentage of sperm that is still motile after cryopreservation varies from 0 to 25 of the original motile fraction. The duration of cryopreservation has no influence on these percentages.[20]

All of our patients had normal spermatogenesis after vasectomy. After all, this concerned only men who had proved their fertility prior to their sterilisation, in many cases in a relationship with a different woman. Probably all of the CBAVD patients have at least some focal areas with normal spermatogenesis. In the cases of unexplained obstructive azoospermia, sperm was less frequently found by means of PESA, and cryopreservation turned out to be possible with only one third of the men.

In conclusion, a man in the Netherlands who has a wish to father children and who for clinical reasons is supposed to have obstructive azoospermia, who has an epididymis that can easily be felt, and who has normotrophic testicles may be referred to a centre for in-vitro fertilisation where ICSI is carried out in combination with PESA. For all other cases of azoospermia, no treatment with a man's owns sperm is possible in the Netherlands. In case of an unknown cause, there is a lower probability of retrieval of epididymal sperm than in those cases when the cause is known, but the difference between the two groups is statistically not relevant. This information is important for the counselling of couples that consider assisted reproduction.

Meanwhile, based on the first draft of this article, an amendment to the protocol approved by the CCMO was passed. This amendment states that testis biopsies must no longer be carried out if epididymal sperm was found by means of "diagnostical" PESA.

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Chapter 6

Obstructive azoospermia in men who wish to father children; initial clinical results of intracytoplasmatic sperm injection (ICSI) with surgically retrieved epididymal sperm

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Abstract

OBJECTIVE: To evaluate the results of intracytoplasmatic sperm injection (ICSI) with surgically retrieved epididymal sperm.

DESIGN: Prospective, descriptive.

METHODS: Patients with an obstructive azoospermia confirmed by cytological examination of a testis biopsy, and conforming to the regular IVF/ICSI criteria as laid down in 2001 at the University Medical Centre St Radboud Nijmegen, the Netherlands, were included for percutaneous epididymal sperm aspiration (PESA) and ICSI. The main outcome measure was the ongoing pregnancy rate per initiated cycle.

RESULTS: A total of 44 cycles were started in 31 couples. In 43 cases (98%) an ovum pick-up was performed and 41 (93%) embryo transfers were carried out. In 19 (43%) cases, treatment resulted in a positive pregnancy test and 15 (34%) ongoing pregnancies were recorded. In total, 17 healthy children were born (11 boys and 6 girls).

CONCLUSION: ICSI with surgically retrieved sperm was successfully used as a treatment for childlessness caused by obstructive azoospermia.

Introduction

Intracytoplasmatic sperm injection (ICSI) with surgically retrieved sperm has been permitted again under certain conditions in the Netherlands since 1 January 2001. During the previous years, for Dutch couples, this treatment had only been possible abroad. Since 1994, in the Netherlands ICSI was carried out either with ejaculated sperm or with surgically retrieved sperm. In the latter case, the sperm was retrieved from the epididymis by means of microsurgical or percutaneous epididymal sperm aspiration (MESA or PESA, respectively), or from the testis by means of testicular sperm retrieval (TESE).

In 1996, the Dutch Association of Obstetrics and Gynaecology (NVOG, Nederlandse Vereniging voor Obstetrie en Gynaecologie) and the Association of Clinical Embryologists (KLEM, Vereniging van Klinisch Embryologen) established a moratorium on ICSI with surgically retrieved sperm because of the uncertainty about its consequences for posterity. It was unknown what the effect would be of the aging of the sperm cells that for a longer time had been stored up in the epididymis, or what effect immature sperm cells from the testis would have. In 1998 the moratorium was taken over in the Planning Decision Concerning In-Vitro Fertilisation.[1]

Until so far, it does not appear from any research in any other country that ICSI combined with MESA, PESA or TESE would result in a larger number of congenital defects in posterity.[2,3,4] In studies carried out at our own IVF laboratory, motile epididymal sperm cells did not show any increased damage on the DNA either.[5,6] As a consequence, the NVOG, the KLEM, the Dutch Urology Association (Nederlandse Vereniging voor Urologie), and the Minister of Health, Welfare and Sports proposed to repeal the moratorium under certain conditions. By 1 January 2001, the Minister ordered the removal of the moratorium and modified the planning decision in such a way that the use of surgically retrieved sperm cells from the epididymis (by means of MESA or PESA) was permitted in a protocol that was tested by the Central Commission for Human-Centered Research (Centrale Commissie Mensgebonden Onderzoek: CCMO).[7]

The Radboud University Nijmegen Medical Centre is the first medical centre in the Netherlands that reintroduced ICSI with surgically retrieved sperm, in conformity with the protocol approved of by the CCMO. Criteria are described in the protocol, according to which only men with an obstructive azoospermia may be admitted to the treatment. Moreover, in this protocol the follow-up research into the physical and psychological condition of the children born after the treatment is described.

In the meantime, the MESA technique in Nijmegen was largely replaced by PESA, as the latter is an easier technique with a similar output and that may be carried out as a policlinical operation.[8] In this article we will describe the first clinical results after one year of PESA-ICSI-treatment, this means all of the cycles started in 2001.

Patients and methods

After the moratorium had been instituted in 1996, all patients at the Radboud University Nijmegen Medical Centre eligible for ICSI-treatment with surgically retrieved sperm where registrered on a waiting list. In 2001 they were informed in writing that ICSI-treatment with surgically retrieved sperm was permitted again. The protocol and the subsequent follow-up of the children were explained to the couples. After that, "informed consent" was obtained. As soon as "obstructive azoospermia" had been diagnosed and PESA had been carried out, the couple in question was registered on the waiting list for ICSI treatment. A number of couples from the waiting list already had their child wish fulfilled either by means of MESA or TESE-treatment combined with ICSI abroad, by means of artificial insemination with donor sperm, or by means of adoption. On the other hand, after it had become known that in Nijmegen treatment was possible again, more couples from places outside the district of Nijmegen were referred. In the present analysis, only those couples were included who actually had started their ICSI treatment in 2001.

Criteria for inclusion and exclusion

The main criterion for inclusion was the existence of a histologically proved obstructive azoospermia that could not possibly be cured by means of surgery. The exclusion criteria for in-vitro fertilisation (IVF) and ICSI that are customary at our hospital were maintained, i.e. woman's age of 42 years or more, woman's age of 40 or 41 years, combined with a basal (on the first three days of the cycle) follicle-stimulating-hormone (FSH) level

> 10 U/l, or basal FSH-level > 20 U/l for women aged less than 40 years. Furthermore, couples were excluded from treatment if the woman was either seriously overweight or seriously underweight, if they had serious psychosocial problems, or if either the man or the woman was seropositive for HIV or for the Hepatitis-B-virus.

Genetic examination

Men with a congenital bilateral absence of the ductus deferens (CBAVD) were examined as to whether they were carriers of genetic mutations for cystic fibrosis.[9,10]

PESA-procedure

In those cases when cryopreservation of sperm retrieved during PESA had been impossible (because after defrosting testing no motile sperm cells had been found), but motile sperm cells had been observed during direct assessment, the ICSI-procedure was started all the same, and the PESA-procedure was repeated on the day of the ovum retrieval (a so-called acute PESA).

ICSI-procedure

The woman's treatment did not differ in any way from the regularly procedure for IVF or ICSI: suppression of the emission of luteinising hormone and FSH by the hypophysis with the aid of gonadotrofine-"releasing"-hormone (GnRH) agonists, stimulation of the ovaries with recombinant FSH, a last phase of oocyte maturation by means of human choriongonadotrofine (hCG), after which a follicle puncture took place in order to obtain oocytes. Only mature, morphologically normal oocytes were used to inject one motile sperm cell per oocyte with a micropipette through the zona pellucida into the cytoplasma of the oocyte (table).[11] Either defrosted sperm cells from a PESA-procedure carried out at an earlier stage or fresh sperm cells from an acute PESA-procedure were used. If fertilisation had occurred, a maximum of two embryos was inserted into the uterus on the third day after the follicle puncture. Whenever possible, the remaining embryos were cryopreserved (these embryos might possibly be defrosted and inserted into the uterus in a later cycle). The luteal phase was supported with progesterone administered through the vagina.

Fifteen days after the embryo transfer a pregnancy test was carried out. A pregnancy was defined as being a positive pregnancy test in the urine or serum two weeks after the embryo transfer. An ongoing pregnancy was defined as an intra uterine pregnancy with a duration of at least twelve weeks with heart action determined by means of a ultrasound. The primary outcome measure was the number of ongoing pregnancies for each cycle that had been started.

Statistical analysis

Differences between percentages of ongoing pregnancies for each cycle that had been started were considered to be not statistical significant if there was overlapping of the confidence intervals belonging to them.

Results

During the period that was examined, 31 couples had started treatment, with a total of 44 ICSI-PESA-cycles in all. Nine couples had two cycles, and two couples had three cycles. In one cycle, no follicle puncture was carried out because of imminent hyperstimulation. In two cycles, no embryo transfer was carried out: in one case no sperm cells had been obtained during the PESA-procedure, whereas a follicle puncture had been carried out, and in one case only one oocyte was obtained that had an abnormal shape and therefore was unsuitable for injection. Altogether, 511 oocytes were obtained (on average 11.9 oocytes for each follicle puncture, extremes: 1-26 oocytes), 417 of which (82% of the oocytes obtained) could be injected. Fertilisation occurred 276 times: 54% of the oocytes obtained, 66% of the number of oocytes that were injected.

The results of the treatments are presented in Table I. Nineteen pregnancies occurred: four times (21%) there was a non-vital pregnancy, and fifteen times there was an ongoing pregnancy (34% for each cycle that had been started): there were twelve single babies and three twins (20% of the fifteen continuous pregnancies). There were no differences between the results of the first, the second, and the third cycle. Because of the limited period of study, not all eligible couples started a second or a third cycle.

Table I Result per cycle: number of ongoing pregnancies among 31 couples with a child wish and with obstructive azoospermia after intracytoplasmatic sperm injection with surgically retrieved sperm, Radboud University Nijmegen Medical Centre, 2001

	1st cycle	2nd cycle	3rd cycle	total
Number (%)[a]				
Cycles started	31	11	2	44
Follicle punctures	30 (97)	11 (100)	2 (100)	43 (98)
Embryo transfers	29 (94)	10 (91)	2 (100)	41 (93)
Pregnancies	15 (48)	3 (27)	1 (50)	19 (43)
Number (%; 95% -CI)[a] Ongoing pregnancies	11 (36; 19-55)	3 (27; 6-61)	1 (50)	15 (34; 21-50)

[a] The percentages are related to the number of cycles started.
CI = conficence interval

The group could be subdivided according to a number of different causes of obstructive azoospermia (Table II): CBAVD (15 couples, 24 cycles); vasectomy (six couples; nine cycles); trauma or infection (six couples; six cycles); and unexplained cause (four couples; five cycles). There were no statistical significant differences between the groups in percentages of ongoing pregnancies.

Table II Result per indication: number of ongoing pregnancies with 31 couples with a child wish and an obstructive azoospermia after intracytoplasmatic sperm injection with surgically retrieved sperm, Radboud University Nijmegen Medical Centre, 2001

	Cause of obstructive azoospermia			
	CBAVD	Vasectomie	Trauma or injury	Unknown
Number (%)[a]				
Cycles started	24	9	6	5
Follicle punctures	23 (96)	9 (100)	6 (100)	5 (100)
Embryo transfers	23 (96)	9 (100)	5 (83)	4 (80)
Pregnancies	12 (50)	2 (22)	3 (50)	3 (60)
Number (%; 95% -CI)[a] Ongoing pregnancies	10 (42; 22-63)	2 (22; 3-60)	2 (33; 4-78)	2 (40; 5-85)

[a] The percentages are related to the number of cycles started.
CI = conficence interval

An acute PESA was carried out eighteen times in all (one cycle was cancelled before the follicle puncture because of imminent hyperstimulation and in one case no sperm was retrieved); in the remaining 25 cycles cryopreserved sperm was used (table III). The percentages of ongoing pregnancies for each cycle that had been started after using fresh sperm or cryopreserved sperm were comparable: 36% (7/25) versus 32% (6/19). The four non-vital pregnancies occurred after using cryopreserved sperm (twice in the case of CBAVD, once after an accident or an infection, and once due to an unexplained cause).

Table III Number of cycles started and ongoing pregnancies with 31 couples with a wish to father children and an obstructive azoospermia after intracytoplasmatic sperm injection with surgically retrieved sperm, Radboud University Nijmegen Medical Centre, 2001, using cryopreserved sperm or fresh sperm

	Cause of obstructive azoospermia				
	CBAVD	Vasectomie	Trauma or injury	Unknown	Total
Cryopreserved sperm					
Cycles started	8	8	7	2	25
Ongoing pregnancies (%; 95% -CI)[a]	4 (50)	2 (25)	2 (29)	1 (50)	9 (36; 18-58)
Fresh sperm					
Cycles started	15	1	0	3	19
Ongoing pregnancies (%; 95% -CI)[a]	5 (33)	0 (0)	0 (0)	1 (33)	6 (32; 13-57)

[a] The percentages are related to the number of cycles started.
CI = conficence interval

From the fifteen pregnancies that occurred, seventeen children were born: eleven boys and six girls. No congenital defects were diagnosed with any of these children. All of the couples had been offered prenatal diagnostics; seven of them did not make any use of it, and seven couples had an extensive ultrasound carried out in about the 20th week of the pregnancy (no congenital defects were found). One couple with a twin pregnancy had an amniocentesis, because of the woman's age. In one foetus a partial trisomy 9 (as a result of a small duplication of a piece of the long arm of one of the chromosomes 9) was diagnosed. In the 21st week of the pregnancy the latter was reduced to a single pregnancy that subsequently took an uncomplicated course and resulted in the birth of a healthy daughter.

Discussion

In this study, ICSI with surgically retrieved epididymal sperm was found to be an effective treatment for couples with unvoluntary childlessness based on obstructive azoospermia. The fifteen continuous pregnancies that resulted from the 44 cycles that had been started yielded a percentage of 34 (95% - BI: 21-50), which is comparable to the success percentages at our hospital for ICSI-cycles with ejaculated sperm (28%; 24-32) (www.nvog.nl/files/ivfcijfers2001perkliniek.pdf). The relatively small scale of the study produces broad intervals of reliability, so that it is difficult to draw any clear conclusions.

During the same period, the average age of the women who had a follicle puncture carried out for ICSI-treatment with ejaculated sperm was 32.9 years, and thus is comparable to the average age of 32.7 years in the study group. Moreover, the success percentage is comparable to that in foreign studies, in which the percentage of ongoing pregnancies after an ICSI-PESA-treatment or an ICSI-MESA treatment with obstructive azoospermia varies from 28.6% to 48.5%.[12,13,14]

Fertilisation of oocytes in our group which is 66% is comparable with foreign studies, which includes that the percentages of fertilisation of the oocytes obtained vary from 53% to 72.4%.[12,15,16] Moreover, these percentages were comparable to the percentages of fertilisation in the group with whom an ICSI-cycle with ejaculated sperm was started at our hospital in 2001. 73% of the oocytes thus obtained were fertilised.

There were no differences between the percentages of onoing pregnancies after ICSI-treatment in combination with an acute PESA, compared to the percentages in the case when cryopreserved sperm was used. This also turned out to be the case in some foreign studies.[15,16] If it is possible to use cryopreserved sperm, this has some advantages for the patient (only one operation is necessary and one has the certainty to have vital sperm at the moment of the follicle puncture) as well as for the logistics of the treatment (no acute PESA is necessary). However, if no sperm can be cryopreserved, then it is a good alternative to carry out an acute PESA. In this context it is essential that there is good cooperation between the IVF Department and the urologists.

In one pregnancy, a partial trisomy 9 was diagnosed by means of an amniocentesis; this probably was a chromosomal de-novo anomaly. It is not clear whether this diagnosis was based on coincidence, or whether it was due to the ICSI-treatment or to the PESA-procedure. Recently, a higher percentage of chromosomal de-novo defects with ICSI-children was reported, indicating that it remains necessary to pay attention to this aspect.[17] A good follow-up of the children is necessary, meaning that a physical as well as a psychological examination of the children will be carried out at the age of 2 and 6 years. By means of these examinations, we intend to examine, as is being done in research abroad, whether ICSI-PESA-treatment is not only a successful method but also a safe method without consequences for posterity. This new opportunity for patients with obstructive azoospermia who wish to father children as an alternative to adoption, to artificial insemination with donor sperm, or to treatment abroad.

Besides the UMC in Nijmegen, and recently the UMC in Utrecht and the MC in Rotterdam, various other hospitals will begin to carry out the ICSI-PESA-protocol, so more patients may receive treatment in their own part of the country.

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Part 2

Follow-up after ICSI with non-ejaculated sperm

Chapter 7

Constitutional DNA copy number changes in ICSI children

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Abstract

BACKGROUND: Over the last three decades, technological developments facilitating assisted reproductive techniques (ART) have revolutionized the treatment of subfertile couples, including men suffering from severe oligospermia or azoospermia. In parallel with the advent of these technologies, there is a great concern about the biological safety of ART. This concern is supported by the clinical observation that the frequency of congenital malformations is slightly elevated among ART-conceived children.

METHODS: In this explorative study, we have used tiling-resolution BAC array-mediated comparative genomic hybridization to investigate the incidence of de novo genomic copy number changes in a group of 12 ICSI children, compared with a control group of 30 naturally conceived children.

RESULTS: In 6 of the 12 ICSI children, we found 10 apparently de novo 'same direction genomic copy number changes' [i.e. simultaneous copy number gain (or loss) with respect to both biological parents], notably losses. In statistically significant contrast, similar observations were encountered only six times in the control group in 5 of the 30 children. However, our study group was small, so a larger group is needed to confirm these findings.

CONCLUSIONS: Loci at which we found de novo alterations are known from the human genome database to be prone to large DNA segment copy number changes. As discussed, various molecular mechanisms, including the consequences of delayed male meiotic synapsis and replication fork stalling at early embryonic cell cycles, might trigger these copy number changes.

Introduction

In Europe, in 2002, the clinical pregnancy rate for ICSI was 29.4% per transfer, and it has been estimated that 1–3% of the overall number of live births are the result of ICSI.[1] These figures clearly demonstrate that ICSI is currently being successfully applied on a large scale. Even men with azoospermia often have the possibility to father their own genetic progeny, through ICSI with surgically retrieved sperm at the level of the epididymis or testis.

In parallel with the advantage of these technological developments, there is an observation that ICSI seems to be associated with slightly elevated incidences of certain birth defects. Large follow-up studies by several independent research groups have revealed a small though statistically significant increase in the incidence of certain birth defects in IVF and ICSI children compared with naturally conceived infants.[2,3,4] In theory, this increased risk may be caused by several factors which may either act alone or in conjunction.[5,6] In the first place, male gamete-associated risks: sperm carrying DNA anomalies, i.e. breaks, aneuploidy, Y chromosome deletions or structural changes, may be transferred.[7,8,9] Second, there are risks contributed by the female gamete: the injection of the oocyte itself may cause damage to the ooplasm or meiotic spindle apparatus, and its DNA repair status may determine the mutagenic outcome of sperm DNA lesions.[8,10,11,12] Suboptimal female gametes, which otherwise would have been bypassed by natural selection, may be fertilized due to ovulation induction.[13] Indeed, it is beyond any doubt that a significantly higher rate of de novo chromosomal anomalies such as sex chromosome aneuploidies and structural chromosome anomalies, notably reciprocal translocations, has been observed in ICSI-mediated offspring.[14]

This has led to a situation in which we do not know to what extent assisted reproductive techniques (ART) increase the genetic load, how epigenetic aspects my be involved as well and how this could be reflected in the expressed congenital malformations. Lack of knowledge regarding the primary molecular mechanism(s) underlying the observed ICSI-associated increased rate of birth defects has thus far prevented further technical innovation for optimalization of the procedure.

Earlier results from array-based whole genome profiling in the fertile population have already firmly established that genomic duplications and deletions in the size range of a few kilobases to several megabases are relatively common.[15,16,17] These duplications and deletions are not necessarily translated into readily recognizable phenotypic changes, although a role in human evolution has repeatedly been suggested.[18,19] At the same time, de novo regional duplications, deletions and inversions, triggered by genomically unstable sequences, involving ectopic homologous recombination are well-established causes for a still growing number of genetic diseases.[20]

In the present explorative study, we used bacterial artificial chromosome (BAC)-array mediated comparative genomic hybridization (array-CGH) analysis in order to identify constitutional de novo DNA copy number changes, including those that are phenotypically silent. With DNA copy number variation (CNV), two or multiple copies are arranged in tandem and/or there may be more elaborate patterns such as those caused by segmental duplications, where the numbers of copies are highly variable.[17] In contrast to most of the other whole genome profiling platforms that were available at the time the study was performed, the tiling-resolution BAC arrays used provide unbiased genomic coverage, including polymorphic genomic intervals (comprehensively listed at the 'Database of Genomic Variants', http://projects.tcag.ca/variation/), at a practical resolution of around 100 kb.[21]

Through the use of BAC arrays, it is possible to detect DNA changes (i.e. large-scale copy number changes) more efficiently than by using the single nucleotide polymorphism (SNP)-arrays which were also available at the time this study was performed, because with the latter the CNV intervals were systematically underrepresented.[22,23] This is of considerable importance since our data strongly suggest that copy number changes of large polymorphic intervals are frequently targeted in ICSI-children.

Materials and Methods

Study design

This explorative study was performed with prior written informed consent of both parents and with approval of the Institutional Review Board of the Radboud University Nijmegen Medical Centre. Blood samples were obtained from both parents and from the umbilical cord of the child

immediately after delivery. Six children (four boys and two girls) were born after ICSI with ejaculated sperm from fathers suffering extreme oligoasthenoteratozoospermia (OAT) in which no Y chromosome microdeletions or chromosomal abnormalities were found. Six additional children (two boys and four girls) born after ICSI with sperm from a percutaneous epididymal sperm aspiration (PESA) procedure had fathers with obstructive azoospermia [two times congenital bilateral absence of vas deferens (CBAVD) and four times failed vasovasostomy].

Relative genomic copy number profiles of all children were determined by separate comparison with both parents.

As a reference, we used genomic copy number profiles from 30 trios (child and biological parents), which were produced in the context of a previous departmental research program,[24] all without any apparent link to reduced fertility. In this study, the proband and both parents were individually compared with a control DNA reference pool containing equal amounts of peripheral blood-derived DNA from 10 random individuals, enabling a subsequent mathematical establishment of relative copy number changes.

Tiling-resolution array-based CGH

In this study, we have used a tiling-resolution genomic microarray consisting of 32 447 overlapping BAC clones selected to cover the entire human reference genome,[21,25] which was prepared as previously described.[24,26] In short, genomic target DNAs were isolated from bacterial cultures using an AutogenPrep 960 (Autogen, Holliston, MA, USA) according to the manufacturer's instructions. Subsequently, ~50 ng DNA from each of the clones was amplified using degenerate oligonucleotide-primed PCR (DOP-PCR), then dissolved at an average concentration of 1 µg/µl in 30% DMSO-containing spotting buffer, and spotted onto CMT-ultragaps coated glass slides (Corning) using an Omnigrid 100 arrayer (Genomic Solutions).

Labeling and hybridization

DNA was extracted from umbilical cord derived (newborn) or peripheral blood (parents and individuals from control group) using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's procedure with minor modifications. In brief, DNA was isolated after Proteinase K and RNase treatment. Two washing procedures were performed with washing buffer AW2. DNA was eluted in AE buffer. Subsequent hybridizations were performed basically as described previously.[27] In brief, 500 ng of genomic

DNA from the umbilical cord blood sample and that of one of the parents was labeled by random-primed labeling with Cy3-dUTP or Cy5-dUTP (Amersham Biosciences), respectively. Labeled DNAs were mixed with 120 µg human Cot-1-DNA (Roche), co-precipitated and re-suspended in hybridization solution (50% formamide; 10% dextran sulfate; 2 x sodium saline citrate (SSC); 4% SDS; 10 µg/µl tRNA (Invitrogen). Slides were hybridized for 18 h at 37°C with active re-circulation of hybridization fluid, followed by five wash cycles in 50% formamide, 2 x SSC at 45°C and five wash cycles in phosphate buffer at 20°C. Subsequently, slides were dried by centrifugation and scanned on a GenePix Autoloader 4200AL laser scanner (Axon Instruments, Union City, CA, USA). Analysis of the microarray image files was performed with the GenePix Pro 5.0 software package (Axon Instruments), and all data were uploaded into a local database expert system for automated data-normalization and further analysis.

Data processing and statistical analysis

Genomic intervals showing altered test/reference (T/R) fluorescent intensity values, and as such qualifying as candidate regions for putative de novo copy number alterations, were detected by expert eye, with the standard aid of hidden Markov modeling (HMM) as described previously.24 Initial statistical analysis was performed using Fisher's exact test. A statistical significant difference was defined as $P < 0.05$. All identified genomic copy number alterations were compared with both public and private databases of known disease-unrelated large-scale CNVs (http://projects.tcag.ca/variation/). Gene organization of the regions involved was obtained from public databases (mainly: http://genome.ucsc.edu NCBI genome build 36.1, March 2006).

Pyrosequencing

In order to further evaluate the array-CGH findings, and in an attempt to determine absolute genomic copy numbers for selected genomic intervals in both children as well as their biological parents, pyrosequencing (PSQ) reactions were performed for multiple SNPs residing within these genomic regions. PSQ was selected as the most appropriate technological approach as its dynamic detection-range extends well beyond that offered by other approaches, for instance, MLPA (L.E.L.M. Vissers, personal communication). Interphase fluorescence in situ hybridization (FISH) analysis was considered not to be appropriate as the previously established limited genomic distance of some of the repeat-intervals under

investigation was assumed to impair reliable copy number counts due to fluorescent signal-merging. PSQ (for an explanation of the technique, see http://www.pyrosequencing.com) was performed according to the protocol of the manufacturer, with minor modifications.[28] Briefly, ratios of SNP genotypes were determined using a Pyrosequencer PSQ96MA platform (Biotage AB, Uppsala, Sweden). SNP-specific PSQ primer combinations were developed in-house, using dedicated 'PSQ Assay Design' software (Isogen Life Science). Primers were ordered from Biolegio BV, Nijmegen, The Netherlands, or Isogen Life Science, IJsselstein, The Netherlands. SNP-specific PSQ primer sequences are available from the authors upon request. PSQ reactions were performed in triplicate.

Copy number estimations

Genomic copy number estimations were mainly based on observed SNP genotype ratios. In addition, the number of copies apparently present within the human reference genome was estimated through BLAT searches at the UCSC Genome Bioinformatics site (http://genome.ucsc.edu/cgi-bin/hgBlat), using sequences (typically a few hundred bases) encompassing the SNP under investigation (for an example see lower panel of Fig. 1, which reveals that SNP RS16875985 appears to be present in seven copies per haploid reference genome). Arbitrary 'reference copy numbers' obtained in this way were used as initial offsets to establish 'best-fit copy numbers' in ICSI children and their parents. In addition, also average, region-specific T/R values (Fig.1 upper panel) were taken into consideration.

Results

One year after birth, all parents of the study group were sent detailed questionnaires in order to establish the medical condition of their children. In addition, children born after ICSI with sperm from a PESA procedure went through an extensive medical examination at the age of 2 years in the context of standard national ICSI-PESA guidelines. The mean duration of pregnancy of the study group was 39^{+3} weeks (range: $36–42^{+1}$). Their mean birthweight was 3578 g (range: 2610–4320). One child was part of a twin pregnancy with duration of 36 weeks and a birthweight of 2610 g (the other part twin was not included in the study group, but was also healthy). None of the children had a minor or major malformation, and none was admitted at the hospital in the first year of life.

Array-CGH

Using a HMM algorithm with standard settings, as explained in more detail in the Materials and Methods section, genomic copy number differences with respect to a single parent could easily be identified. The large majority of these changes, however, seemed to represent common variations in copy number (http://projects.tcag.ca/variation/), which are known to be fully compatible with normal Mendelian inheritance. It is therefore reasonable to assume that the large majority of these are unlikely to represent de novo genomic copy number changes.

In a number of instances, 'same direction copy number changes' [i.e. simultaneous copy number gain (or loss) with respect to both parents] were identified. There were 10 such genomic intervals in six children detected among the ICSI children (n =12). All these intervals belonged to a previously defined set of 13 most frequently encountered regions of genomic polymorphism.[17,29] Scanning of the control group of 30 child–parent trios for similar 'same direction copy number changes' in these intervals revealed six similar events in five children (Table I).

A representative example of one such interval (targeting chromosome 5q12, family K) is shown in Figure 1. For this case, the BAC array (upper panel) shows a clear copy number gain of the newborn compared with both the father (plotted in green) and the mother (plotted in red).

Pyrosequencing

On the basis of available HapMap data (http://www.hapmap.org/) SNP-linked PSQ primers were designed and optimized for a total of 14 loci, representing five of the seven genomic intervals listed in Table I. No informative SNPs could be identified for the 0.5 Mb interval on chromosome 22q11. Primer-combinations and optimized PCR-/PSQ-conditions are readily available upon request from the authors. Primary data-processing using standard PSQ96MA software (Biotage AB), yielded reproducible SNP genotype ratio calls for all the SNPs typed (primary experimental data are available upon request). Figure 2 shows a composite figure containing representative examples of the graphical output produced by the PSQ96MA software package (family K).

Table I Same direction copy number changes (target regions)

	Chr. 5q13.2 (68,75-70,75 Mb)	Chr. 8q21.2 (86,63-86,92 Mb)	Chr. 10q11.2 (47,08-48,13 Mb)	Chr. 14q11.1 (18,43-19,39 Mb)	Chr. 15q11.2 (19,08-19,99 Mb)	Chr. 19p13.2 (8,65-8,70 Mb)	Chr. 22q11.1 (14,30-14,81 Mb)
SNPs used for PSQ[a]	RS16875985	RS7388011 RS987390 RS6981582	-	RS915672 RS543617 RS7153371	RS1112179 RS925313 RS2672358	RS4804079 RS301420 RS301424	nothing found
Family ID	**ICSI children**						
A[b]		loss				loss	
B[b]	loss				loss		
C[b]					loss		
D[b]							
E[b]							
F				loss		loss	loss
G							
H	loss						
K[b]	gain						
M							
N							
O							
	Controls[c]						
96	loss						
58				gain			gain
350					loss		
117					loss		
17			gain				

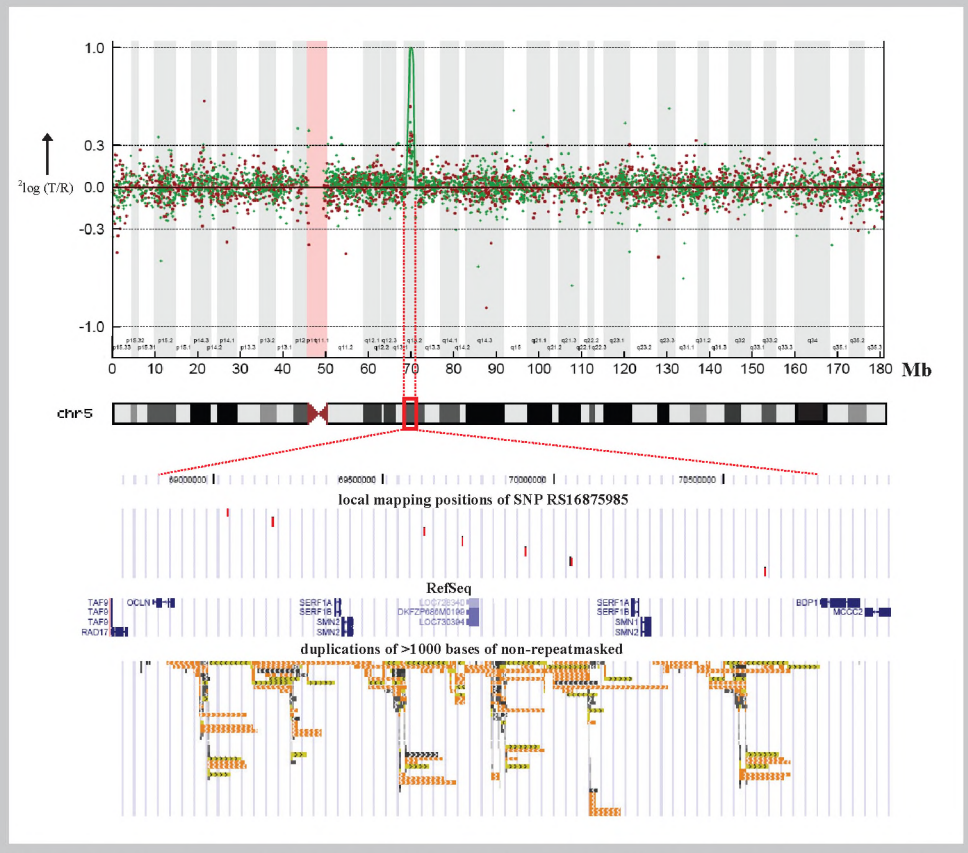
[a] PSQ: pyrosequencing
[b] child born after ICSI with sperm from a PESA
[c] only control families (n=30) in which 'same direction copy number changes' were observed are listed

Statistical analysis

There were 10 'same direction copy number changes' detected among six ICSI children (total number tested: 12), whereas only six similar genomic regions in five children could be detected within our much larger (n = 30) control group (Table I). The two tailed P-value by Fisher's exact test for these values equals 0.049, so 'same direction copy number changes' are significantly more frequent within our panel of ICSI children than among children from our control group. Although the numbers are small, in the ART group (n = 10), all but one were losses, whereas for the control group (n = 6) there was a balance between losses and gains (Table I). Hence, when testing for losses only, a higher level of significance is obtained (P = 0.031).

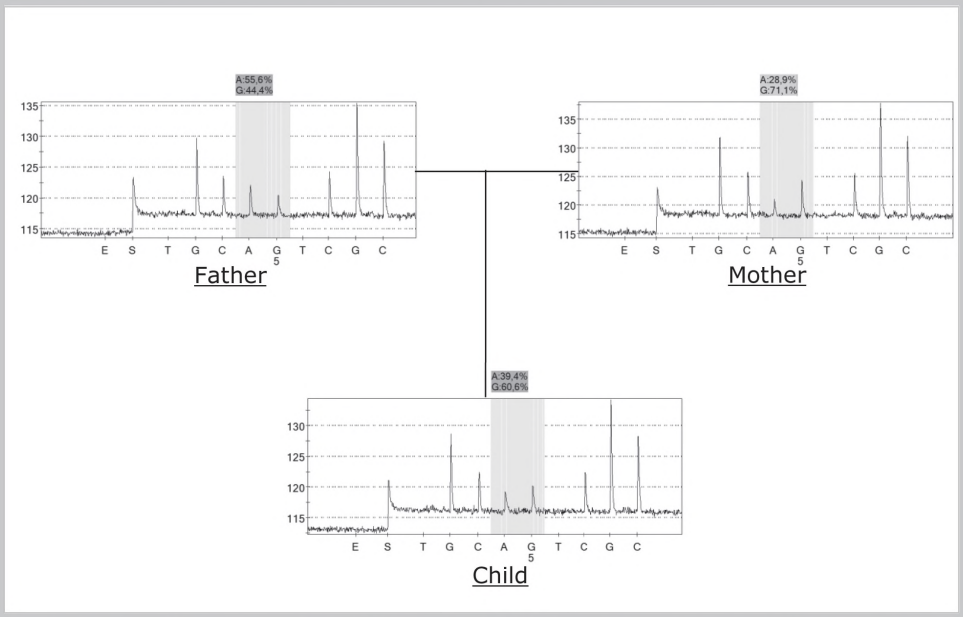
There were six copy number changes found in four children (out of six) born after ICSI with sperm from a PESA, and four changes in two children (out of six) born after ICSI with ejaculated sperm from fathers suffering extreme OAT. The two-tailed P-value by Fisher's exact test equals 0.567, indicating that there is no significant chance difference for having 'same direction copy number changes' between children born after ICSI with sperm from a PESA and children born after ICSI with ejaculated sperm from fathers suffering extreme OAT.

Figure 1 Same direction copy number change in family "K" (at 5q13.2).



Upper panel: representative example of a "same direction copy number change" in family K (Table 1). 2log(T/R) values of child versus father (in green) and child versus mother (in red) are plotted in a superimposed manner. A region with significant T/R value differences, as detected by the HMM algorithm (green line) is clearly visible around the 70 Mb position on chromosome 5q13.2. Middle panels: mapping positions of nucleotide sequence surrounding a representative SNP (RS16875985) as identified through a standard BLAT search at the Human Genome Browser website are projected, together with the mapping position of well established protein encoding genes residing in this genomic interval. Lower panel: duplicated intervals identified in the reference human genome sequence, and exceeding 1 kb in size reveal the complex genomic architecture of this particular genomic interval.

Figure 2 Haplotype ratios for SNP RS16875985 (family K)



Single Nucleotide Polymorphism (SNP) RS16875985 (residing at 5q13.2 [Figure 1 middle panel]) haplotype ratios for family K [Figure 1], as determined by pyrosequencing. Standard output format as produced by the PSQ96MA software package is shown. The father, mother and child show ratios compatible with minimal "best fit genomic copy numbers" of 9, 7, and 5 (as shown "ratio-percentages" (shaded boxes) appear to be multiples of 11.1% [one ninth], 14.3% [one seventh] and 20% [one fifth]), respectively. As the haploid human reference genome contains 7 copies (i.e. 14 copies in total), we assume the parents to have 18 and 14 copies respectively. As the child appears to have a T/R value of roughly 1,3 (to 1,5) compared to both parents (Figure 1), the child is supposed to actually have something in the order of 4 (or 5) times the minimal genomic copy number of 5 (i.e. 20-25) copies.

Discussion

As mentioned in the Introduction, large follow-up studies by several independent research groups have revealed a small though statistically significant increase in the incidence of certain birth defects in ICSI children compared with naturally conceived infants.[2,3,4] Also a significantly higher rate of de novo chromosomal anomalies has been observed in ICSI-mediated offspring.[14] These data suggest that ART procedures increase the genetic load. Such an increase, if present, could, besides gross chromosomal changes including CNV of large kilobyte range sequences, also apply to other mutation types such as simple sequence repeat instability, small deletions, duplications, inversions and base pair changes. At the moment, we would not know to what extent an increased genetic load might manifest itself in congenital malformations. The lack of knowledge regarding the primary molecular mechanism(s) underlying the observed ICSI-associated increased rate of birth defects, prompted us to initiate the study described here. We used BAC array mediated CGH (array-CGH) analysis in order to identify possible constitutional de novo DNA copy number changes in ICSI children, which can be detected with the 32k array with a resolving power of around 100 kb. Up to now, the considerable CNV detected with this type of technology at first observation appears to be phenotypically silent, although selective pressures must have been operative.[18,19]

There were 10 'same direction copy number changes' [i.e. simultaneous copy number gain (or loss) with respect to both parents] found in 6 out of the 12 ICSI children, 9 of which were losses. In an attempt to obtain a more accurate basis for our copy number estimates, we subsequently used PSQ as an independent technology platform to validate our array-CGH findings. PSQ data were obtained for all child–parents trios with copy number changes (except for an apparent de novo copy number change at chromosome 22q11.1, for which no suitable SNPs could be selected) and were fully compatible with our BAC array-based observations. As could be expected, PSQ does not provide the investigator with exact copy number counts. The haplotype ratios obtained through this technique, however, can be very helpful nevertheless, as they provide a kind of mathematical framework (i.e. 'multiples of ...', as explained in the legend to Figure 2) for copy number estimates. It should be realized, however, that the ratios obtained by this technique can be explained either by a minimal ratio-compatible number of copies or a multiple of this number, thereby

always providing the mathematical opportunity for a standard Mendelian segregation fit (assuming that copy numbers are not limited). Moreover, this technique is unsuitable for determining the distribution of these copies over the chromosome pair (which would require more extensive segregation analysis or combined sperm- and oocyte-typing).

Since no information can be obtained regarding the distribution of individual copies over the two autosomes, we decided to use statistics to investigate whether the frequency at which 'same direction copy number changes' were encountered in our patient population was significantly different from that found in our reference population. The two-tailed P-value by Fisher's exact test indicated that 'same direction copy number changes' appear to be significantly more frequent within our panel of ICSI children than among children from our control group ($P = 0.049$). This result is more striking when only repeat losses are considered ($P = 0.031$). No significant difference was found for 'same direction copy number changes' in children born after ICSI with sperm from a PESA compared with children born after ICSI with ejaculated sperm from fathers suffering extreme OAT ($P = 0.567$). Our study group was small, so a larger group is needed to confirm these findings.

A Mendelian explanation of our results would assume appreciable size differences between the two 'alleles' from both parents. The reason for ART in these couples was a male infertility problem. Moreover, the association between allele size difference and infertility is at least questionable because of the absence of a theoretical/genetic explanation in the literature. So the de novo occurrence of a CNV, notably losses, in the male germline is the most plausible explanation.

In PESA patients, spermatogenesis is assumed to be normal. However, de Boer et al.[30] found indications for altered kinetics of meiotic prophase in this class with leptotene and late zygote spermatocytes being overrepresented. This is suggestive for difficulties in initiating and finalizing meiotic synapsis and hence crossing-over.[31] Recently, a decreased efficiency at meiotic synaptic initiation and finalization has also been found in cases of human azoospermia.[32]

This observation suggests a link between the increased occurrence of CNV and a disturbed kinetics of first meiotic prophase, likely involving the organization of the axial lateral elements of the synaptonemal complex,

as this organization determines which homologous sequences are brought together in early and late recombination nodules.[33,34]

Impaired progression at the end of zygotene is most prominent at the large heterochromatic blocks that are often different in size between homologues, adjacent to the centromeres of chromosomes 9 and 1.[35] Recently, it has been found that delayed synapsis in these regions also can show as a recombination effect in trans on chromosome 5,[32] where we found CNV at 5q13.2 most frequently.

Alternatively, the origin could be at the elongated spermatid and sperm stages as these, due to contracted chromatin are regarded as devoid of DNA repair, that is subsequently triggered in the zygote. Double strand DNA repair by non homologous end joining and homologous recombination is active in the zygote,[11,12] and a male-female interaction at this level for the generation of paternal Retinoblastoma mutations has been proposed.[10] In this scenario, stalled replication forks in the paternal pronucleus by compromised homologous recombination repair[12] would lead to the mainly deletions in large scale repeat areas.

An extension of the explanation of our results has recently been offered by CNV analysis of phenotypically concordant and discordant monozygotic twins.[36] Somatic mosaicism was shown to be common in peripheral blood-derived DNA, both by a tiling 32K BAC array and by an Illumina system SNP analysis. Hence, the origin of the mutation event, likewise connected with repeat replication stalling, could also be later than the first cleavage division, with later origins expected to contribute less of a CGH effect, if not outweighted by the size of the event.

As far as can be judged by medical examination of the young children of concern here, the genomic changes observed do not have a phenotypic consequence. Because of the small sample size, phenotypic effects were not expected. However, as repeat areas are not by definition genetic deserts (see for instance Figure 1) and genes in these and comparable areas have been linked to phenotypic effects, dosage effects could be at the basis of congenital malformation. For instance, genomic alterations affecting SMN1 (see Figure 1) are linked to the development of spinal muscular atrophy (http://www.genecards.org/).

Although executed at a small scale, our search revealed a relative abundance of 'same direction copy number changes' in an ART setting, increased by a factor of 5 relative to the control population. Our estimate likely is an underestimate as the two alleles are pooled per individual. Hence, meiotic segregation from one parent can obscure a de novo event from the other parent (for a de novo deletion this would mean the inheritance of a longer repeat array from the other parent).

In conclusion, the recently discovered genome mobility by CNV in the human likely is enhanced in an ART setting, a result that should warrant more detailed investigation into especially the mechanisms and germ cell stages involved.

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Chapter 8

Karyotyping, congenital anomalies and follow-up of children after intracytoplasmic sperm injection with non-ejaculated sperm: a systematic review

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Abstract

BACKGROUND: For men with azoospermia, it is possible to father their own progeny by intracytoplasmic sperm injection (ICSI) with epididymal or testicular sperm. Some studies show that children born after assisted reproductive technology (ART) are at increased risk of birth defects, other studies suggest that there is no extra concern about ICSI children conceived with epididymal or testicular sperm.

METHODS: Studies about the karyotypes of fetuses, congenital anomalies and the follow-up of the children born after ICSI with non-ejaculated sperm were identified by means of a systematic literature search.

RESULTS: Eight relevant studies were identified; two studies reported karyotype, five reported malformations and one reported follow-up of children after ICSI. In total, there were 55 out of 1973 (2.8%) abnormal karyotypes in the ICSI with ejaculated sperm group, 0 out of 31 in the ICSI with epididymal sperm group and 5 out of 191 (2.6%) in the ICSI with testicular sperm group. Major malformations were found in 543 out of 12 377 (4.4%) in the ICSI with ejaculated sperm group, 17 out of 533 (3.2%) in the ICSI with epididymal sperm group and 31 out of 670 (4.6%) in the ICSI with testicular sperm group.

CONCLUSIONS: Although there were no statistical differences, the study groups were small and heterogenic, with a number of potential biases. We therefore recommend a standardized methodology of follow-up studies after ART, with well-defined groups of ICSI with ejaculated sperm, ICSI with epididymal sperm and ICSI with testicular sperm, and a control group of naturally conceived children.

Introduction

Assisted reproductive technology (ART) is nowadays available worldwide and has been practiced successfully on a large scale. About 1–4% of all live births in Europe are the result of IVF or intracytoplasmic sperm injection (ICSI).[1] Hansen et al.[2] showed in a systematic review that children born after ART are at increased risk of birth defects compared with spontaneous conceptions. The increase of birth defects in singletons born after ART might be related with the hormonal treatment for infertility or the procedure itself, but the underlying cause of infertility or its determinants might also play a role.[3]

Azoospermia is present in ~5% of all investigated infertile couples[4] and is found in 10% of male infertility cases.[5] Azoospermia can be divided into two groups: obstructive azoospermia (OA) with a normal spermatogenesis and nonobstructive azoospermia (NOA) as the result of a testicular failure. Since the introduction of ICSI, it is possible for these couples to father their own progeny by using sperm retrieved by percutaneous epididymal sperm aspiration (PESA), microsurgical epididymal sperm aspiration (MESA) or testicular sperm extraction (TESE). PESA and MESA are eligible in cases of OA, TESE in cases of NOA or in cases of an azoospermia with a PESA or MESA.

Concerns about the health of the children born after these techniques have been raised, especially in The Netherlands.[6,7] This led to a moratorium in this country for the application of ICSI in azoospermic men using non-ejaculated sperm from 1996 until 2001. From January 2001, ICSI with sperm retrieved by PESA or MESA in the case of an OA was allowed on the condition that there was a follow-up programme of the children, which was approved by the Central Committee on Human Research (CCMO). From June 2007, ICSI with sperm retrieved by TESE followed, on the same condition.

The goal of this study was to investigate the karyotypes of fetuses, congenital anomalies and the follow-up of the children born after ICSI with epididymal or testicular sperm.

Methods

For this systematic review, we collected from PubMed, EMBASE, Web of Science and The Cochrane Central Register of Controlled Trials all papers published on the health of children born after ICSI with non-ejaculated sperm from 1992 (the year of the publication of the first ICSI child) to June 2008 using the keywords: 'epididymal', 'surgical retrieved sperm', 'surgically retrieved sperm', testicular, 'pesa', 'mesa', 'percutaneous sperm aspiration', 'percutaneous epididymal sperm aspiration', 'microsurgical epididymal sperm aspiration', 'microsurgical sperm aspiration', 'tese', 'tesa', 'testicular sperm aspiration', 'testicular sperm extraction', in combination with 'infertility treatment', 'in vitro fertilization', 'IVF', 'fertilization in vitro', 'fertilisation in vitro', 'intracytoplasmic sperm injections', 'sperm injections, intracytoplasmic', 'ICSI', 'assisted reproductive technique' combined with 'development', 'child', 'child*', 'hospital', 'abnormalities', 'birth defect', 'birth defects', 'follow-up', 'congenital malformation', 'congenital anomaly', 'offspring', 'pregnancy outcome', 'obstetric outcome', 'infant outcome'.

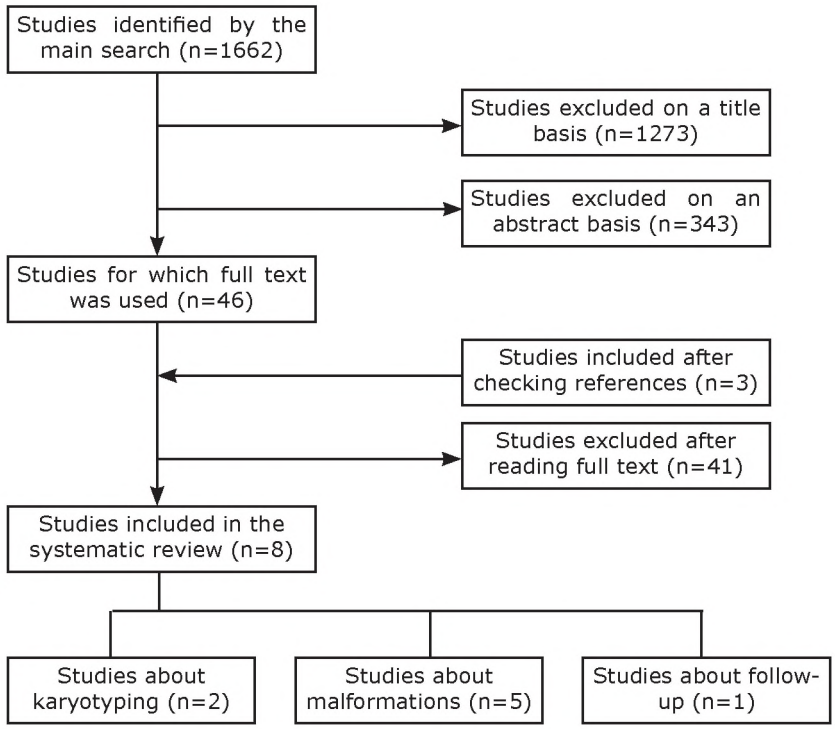
English was used as a limit.

The first and second author selected papers with categorical data of karyotyping of the fetuses and/or congenital anomalies and/or follow-up of the children conceived by ICSI with epididymal or testicular sperm versus ICSI with ejaculated sperm or IVF or naturally conceived children. By reading the titles and subsequently the abstracts, the first selection was made. Case reports, reports describing less than five children and reviews were excluded, but the references of all these articles were checked. If in more than one paper essentially the same group of infants were discussed, we selected the paper with the larger group or with a control group. If it was not possible to discover the origin of the sperm (ejaculated, epididymal or testicular), the article was excluded. For instance, the paper of Wennerholm et al.[8] with a long-term follow-up was not included, because there was a combined group of epididymal and testicular sperm. The first and second author (G.H.W. and D.E.B.) read and selected the studies and extracted data separately. Disagreements were solved in discussion with or without the last author (J.A.M.K.).

Results

The search strategy identified 1662 potentially relevant studies. A flow chart summarizing search results is provided in Figure 1. In the first selection, 1273 articles were excluded, because they did not fulfill the selection criteria, leaving 389 articles. Following further exclusions, nine papers with no data overlap were identified for review, and one was eventually excluded from the final analysis.

Figure 1 Flow-chart for the systematic review



Two studies discussed karyotyping of fetuses.[9,10] There were six studies dealing with congenital anomalies.[11-16] These studies discussed children born after transfer of embryos fertilized with epididymal sperm or testicular sperm. All studies except the study of Fedder et al.[16] discussed children born after ejaculated sperm as well. Four studies had a control group that consisted of IVF children or naturally conceived children, two studies[12,16] had no control groups. Because of the differences between the control groups, we analysed the studies, which described children born after epididymal sperm and after testicular sperm and children born after ejaculated sperm. We used the group with children born after ICSI with ejaculated sperm as the control group. The study of Fedder et al.[16] was excluded from the final analysis, because there was no group of children born after ICSI with ejaculated sperm as a control (comparator) group. They found a relatively high incidence of hypospadias, but no further increased major malformation rate of ICSI with epididymal or testicular sperm compared with IVF children and naturally conceived children as reported in the literature.

Only one study was found with follow-up of the children until 2 years of age.[17]

Finally, there were eight articles included in the systematic review. Of these eight studies reviewed, seven originated from Europe and one from the USA. The earliest study was published in 1998, all the others between 2000 and 2005. The size of the study groups ranged between 504 and 4248 children conceived with ejaculated sperm, between 26 and 198 children conceived with epididymal sperm and between 31 and 229 children conceived with testicular sperm. The characteristics of the remaining studies utilized in this systematic review are described in Table I.

Karyotyping

Two studies discussed the karyotyping of fetuses.[9,10] Table II shows the number of fetuses tested and the percentage of abnormal karyotypes per subgroup. Also the relative risks (RRs) and the 95% confidence interval (CI) are given for abnormal karyotypes per study group compared with ICSI with ejaculated sperm. In the study of Jozwiak et al.,[10] there were different groups of ICSI with ejaculated sperm: cases of male factor, female factor or unexplained factor. In our analysis, we compared only with the group of ICSI in cases of male factor.

In the study of Bonduelle,[9] prenatal diagnosis was performed in 47% of the ICSI pregnancies. In 37% of the mothers tested, there was an age-related risk (maternal age ≥35 years), the others underwent a prenatal test because of a parental chromosomal anomaly or the possible higher risk related to the ICSI procedure, as explained during the genetic counselling sessions. A total of 1586 ICSI fetuses were tested, of which 47 were abnormal: 25 anomalies were de novo (of these, 10 were sex chromosomal anomalies and 15 were autosomal anomalies) and 22 abnormal karyotypes were inherited (17 of these were transmitted through the father). None of the prenatal chromosomal anomalies was found in fetuses after ICSI with epididymal sperm and three anomalies were found in the fetuses after ICSI with testicular sperm. Two of these anomalies were de novo (3.2%) and one anomaly (1.6%) was inherited. Although the values for de novo anomalies were high, the patient numbers were too small to draw valid conclusions. The chromosomal anomalies such as de novo structural anomalies or sex chromosomal anomalies had a relatively benign character. Abnormal karyotypes were also found in 338 karyotyped children at birth, but none of these was after the use of epididymal or testicular sperm.

In the study of Jozwiak et al.,[10] prenatal karyotyping by amniocentesis was recommended universally at 16–20 weeks of gestation for all patients who conceived by ICSI. Only 735 patients of the 1762 (41.7%) underwent amniocentesis, 73% of the mothers were younger than 34 years. They found two (1.5%) abnormal karyotypes (both de novo sex chromosomal) in fetuses after ICSI with testicular sperm. There was no subgroup of fetuses after ICSI with epididymal sperm. No significant difference was found for the frequency of abnormal karyotype between the groups in which ejaculated spermatozoa and testicular spermatozoa were used. Post-natal karyotyping was not described.

Congenital anomalies

Three studies[11,13,14] defined major malformations as structural defects of the body and/or organs, which affect viability and quality of life requiring medical intervention. Kallen et al.[15] excluded congenital malformations that are relatively common, variable in registration, and sometimes associated with preterm birth and low birthweight. The following of such conditions were excluded: preauricular appendix, patent ductus arteriosus at preterm birth (37 weeks), single umbilical artery, undescended testicle, congenital hip (sub)luxation and minor skin malformations (mainly nevus).

Table I Study characteristics by studies of karyotyping, congenital anomalies and follow-up

Authors and publication year	Location	Study design	Study centres	Study groups	Kind of assessment	Age of assessment	Birth years	Maternal age (years) mean ± SD	Total sample size	Percentage multiples[a]	Lost for follow-up
					Karyotyping						
Bonduelle et al, 2002b	Belgium	Prospective cohort study	Single centre	ICSI in subgroups	Prenatal karyotyping by chorionic villus sampling or amniocentesis or postnatal karyotyping	Between 12 and 20 weeks of gestation or at birth	1990-2001	33.5	1563	43.1	53%
Jozwiak et al, 2004	Turkey	Retrospective case control analysis	Single centre	ICSI in subgroups	Amniocentesis	Between 16 and 20 weeks of gestation	1992-2002	31.8±4.4	632	53,9	58.3%
					Congenital anomalies						
Bonduelle et al, 2002a	Belgium	Prospective cohort study	Single centre	IVF and ICSI	Questionnaires and physical examination	At birth and at 2 months	1990-1999	IVF: 32.7±4.3 ICSI: 32.2±4.1	5743	48.3	1.5%
Källén et al, 2005	Sweden	Retrospective population based study	Multi-centre	IVF and ICSI	Diagnostic codes in Swedish Registry of Congenital Malformations and Swedish Hospital Discharge Register	At birth	1987-2001	NA	14 646	NA	NA
Ludwig and Katalinic, 2002	Germany	Prospective control cohort study	Multi-centre	ICSI and natural conceived	Physical examination and ultrasound examination of the kidneys and the hips	Before 8 weeks of age	1998-2000	NA	34 139	39.0[b]	5.4%
Palermo et al, 2000	USA	Prospective cohort study	Single centre	IVF and ICSI	Physical examination	At birth	1993-1999	Ejac.: 36.1±5 Non-ejac.: 34.4±5	3855	41.8	NA
Wennerholm et al, 2000	Sweden	Retrospective cohort study	Multi-centre	CSI in subgroups	Physical examination obtained from medical records	At birth	1993-1998	Epid.: 32.2±4.3 Test.: 32.7±3.7	1034	35.2	NA
					Follow up						
Bonduelle et al, 1998	Belgium	Prospective follow up study	Single centre	ICSI in subgroups	Neurological and psychomotor assessment by a geneticist or paediatrician; a Bayley test	At 2 months, 12 months and 2 years; at ~ 2 years	1991-1995	Epid.: 32.2±4.2 Test.: 32.1±4.3	163	37.4	77% (at 1 year)

[a] percentage multiple children of total study groups
[b] percentage only of ICSI study group
NA= not available

Table II Abnormal fetal karyotypes per study group (%) with RR (95% CI) compared to ICSI with ejaculated sperm

Authors	Abnormal fetal karyotypes			Outcome[a]
	ICSI with ejacul. sperm	ICSI with epidid. sperm	ICSI with testic. sperm	
Bonduelle *et al*	45/1469 (3.1)	0/31 RR: 0	3/63 (4.8) RR: 1.53 (0.49-4.79)	Values for de-novo anomalies were high after ICSI with testicular sperm, but the patient numbers were too small to draw valid conclusions
Jozwiak *et al*	10/504 (1.9)[b]	NA	2/128 (1.5) RR: 0.79 (0.18-3.57)	No significant difference between the groups in which ejaculated spermatozoa and testicular spermatozoa were used

[a] outcome of the study as mentioned in article
[b] in cases of male factor
ejac.= ejaculated; epid.= epididymal; testic.= testicular; NA=not available

Table III Study characteristics by studies of karyotyping, congenital anomalies and follow-up

Authors	Major malformations					Outcome[a]
	ICSI with ejac. sperm	*ICSI with epid. sperm*	*ICSI with testic. sperm*	*IVF*	*Natural conceived children*	
Bonduelle *et al*	84/2477 (3.4)	4/105 (3.8)	6/206 (2.9)	112/2955 (3.8)	NA	No statistical difference (ejaculated sperm vs non-ejaculated sperm; testicular sperm vs epididymal sperm; ICSI vs IVF)
Källén *et al*	139/4248 (3.3)	5/135 (3.7)	3/147 (2.0)	284/10116 (2.8)	NA	No significant difference (between different methods of ICSI; between standard IVF and ICSI)[b]
Ludwig and Katalinic	248/2944 (8.4)	1/26 (3.8)	21/229 (9.2)	NA	2140/30940 (6.9)	No influence of sperm origin; increased risk after ICSI compared with natural conceived children[c]
Palermo *et al*	33/1774 (1.9)	4/198 (2.0)	1/87 (1.1)	30/1796 (1.7)	NA	No difference in frequency (between IVF and ICSI; between ejaculated, epididymal and testicular sperm)
Wennerholm *et al*	39/934 (4.2)	3/69 (4.3)	0/31(0.0)	NA	NA	Similar rate in different subgroups

[a] outcome of the study as mentioned in article
[b] adjusted for potential confounders: year of birth, maternal age and parity, years of involuntary childlessness and maternal smoking in early pregnancy
[c] included still births
ejac.= ejaculated; epid.= epididymal; testic.= testicular; NA=not available

The remaining malformations were classified as 'weeded'. Wennerholm et al.[12] applied the following definition of malformation: any congenital malformation defined in the International Classification of Diseases.[18,19] In the article of Palermo et al.,[11] it was not clear if the major malformations of the study groups were with or without minor malformations. One article[13] described the malformations of live born and stillborn children, as well as of fetuses from spontaneous miscarriages or terminated pregnancies, after the 16th week of gestation. Discrimination for malformations between these groups was not described. In the study, there were 35 (1.0%) spontaneous miscarriages, 18 (0.5%) terminations of pregnancy and 12 (1.4%) stillborns. Three articles[11,12,14] stated the number of stillbirths, but they described only the malformations of the live born children. Palermo et al.[11] report 18 (0.84%) fetal deaths after 20 weeks of gestation and 16 (0.75%) neonatal mortalities. In the study of Wennerholm et al.,[12] there was no perinatal death reported in the epididymal retrieved sperm group (n = 69), and 1 out of 31 in the testicular retrieved sperm group. Bonduelle et al.[14] presented data on stillbirths in the ICSI group of 49 of 2889 (1.7%) births, and in the IVF group, 40 of 2995 births (1.3%). In the ICSI group, there were abnormal findings at physical examination or autopsy in 8 of the 49 children, but the origin of the sperm was not stated. In the IVF group, 2 of 40 stillborn children had congenital abnormalities.

A differentiation for malformations between singletons and multiples was not made in any of the studies. Only one study[15] made an adjustment for year of birth, maternal age and number of infants in birth for comparison of malformations between IVF and ICSI (with differentiation in source of sperm).

Table IV Relative risks (RR) with 95% CI for major malformations of ICSI children with epididymal or testicular sperm compared with major malformations of ICSI children with ejaculated sperm

Authors	RR (95% CI)	
	epid/ejac	*test/ejac*
Bonduelle *et al*	1,12 (0.42-2.98)	0,86 (0.38-1.94)
Källén *et al*	1,13 (0.47-2.72)	0,62 (0.20-1.93)
Ludwig and Katalinic	0,46 (0.07-3.13)	1,09 (0.71-1.66)
Palermo *et al*	1,09 (0.39-3.03)	0,62 (0.09-4.47)
Wennerholm *et al*	1,04 (0.33-3.28)	0

epid=epididymal, ejac=ejaculated; test=testicular

No studies reported statistically significant differences in the rate of major malformations when they compared ICSI children conceived with ejaculated, epididymal or testicular sperm (Table III). In all five studies analysed together, major malformations were found in 543 out of 12 377 (4.4%) in the ICSI with ejaculated sperm group, 17 out of 533 (3.2%) in the ICSI with epididymal sperm group and 31 out of 670 (4.6%) in the ICSI with testicular sperm group. Table IV shows the RR and the 95% CIs of major malformations in children from ICSI with epididymal sperm compared with ICSI with ejaculated sperm and in ICSI with testicular sperm compared with ICSI with ejaculated sperm. In conclusion, the differences in rate of malformations between the children conceived with ejaculated, epididymal or testicular sperm were similar in all the studies regarding their 95% CI. Note that the study of Ludwig and Katalinic[13] deviates in the direction of the estimates compared with the other studies. They found a lower RR for major malformations between ICSI with epididymal sperm (based on only 26 infants) and ejaculated sperm and a higher RR between ICSI with testicular sperm and ejaculated sperm than the other studies. They also found an increased risk of major malformations of ICSI children in comparison with naturally conceived children (data were taken from a prospective population-based birth registry in Germany). It was not clear if this increased risk was related to ICSI with ejaculated sperm or to ICSI with non-ejaculated sperm as well. Furthermore, malformations in stillbirths were included in the major malformations, and finally, although the relative size of the ICSI subgroups are quite different between the five studies, the study of Ludwig and Katalinic[13] deviates most at this point with the smallest group of ICSI with epididymal sperm (n = 26) and a more than eight times larger group of ICSI with testicular sperm. This again may illustrate the diversity in protocols used in the studies.

Minor malformations were mentioned in two of the studies.[11,12] In one study[11], it was not possible to discover the origin of the sperm and in the other study,[12] the minor malformations were distributed evenly in the subgroups with the same incidence as in the general population.

Follow-up

Only one article described the follow-up of children born from ICSI with epididymal or testicular sperm.[17] In the group of children born from ICSI with epididymal sperm, 14 out of 55 children were seen at the age of 1 year for a physical, neurological and psychomotor assessment. There was a large group (77%) lost for follow-up at 1 year and most of the children were still <2 years of age. Two children had a minor developmental problem, one child had an axial hypotonia at 2 months, but was normal at 2 years and one child had a language delay at 2 years. Ten out of 50 children born from ICSI with testicular sperm were seen at 1 year. No problems were described.

Discussion

The goal of this study was to review the literature about abnormal karyotypes, congenital anomalies and the follow-up of the children born after ICSI with non-ejaculated sperm. In doing this, we were aware of the differences in study design and study groups. All studies were cohort or case–control studies, none of them was matched. Some were retrospective, by identifying health registers,[15] and some were prospective, by physical examination[11,12,13] or by physical examination as well as by sending questionnaires.[14] Only one study[15] made adjustment for some variables, which could act as confounders: year of birth, maternal age and parity, years of involuntary childlessness and maternal smoking in early pregnancy.

Identifying the studies, none of them was more methodologically superior or reliable regarding inclusion and exclusion criteria, sample size, statistical adjustment and kind of assessment. The study of Ludwig and Katalinic[13] was superior with inclusion of stillbirths and the study of Kallen et al.[15] was superior with statistical adjustment. Most reliable for kind of assessment was the study of Bonduelle et al.,[14] by sending questionnaires and physical examination. The studies suggest that the risk ratios may be similar in the subgroups of the ICSI procedures. Points of concern are the possible heterogeneity and the low number of children in some subgroups resulting in large CIs. Possible heterogeneity between the study groups in the articles is related to the age of the mother, indication for PESA or TESE, difference in OA and NOA, the use of fresh or frozen-thawed sperm or embryos and difference in percentage of singletons and multiples.

There is a maternal age-related risk (>35 years) for aneuploidy;[20] therefore, it is important to eliminate this possible bias in studies for karyotyping. In the study of Bonduelle et al.,[9] they found 2.2% de novo anomalies in the age-related group (≥34 years) and 1.2% in the group of the fetuses with mothers aged ≤35 years. The study of Jozwiak et al.[10] did not found a significantly correlation between the maternal age and the frequency of abnormal karyotypes: 1.4% abnormal karyotypes in the group mothers ≤34 years and 1.6% in the group mothers ≥35 years. Furthermore, there is still a risk for miscarriage after chorionic villus sampling or amniocentesis.[21] Because of this risk not all women do the test, especially considering the long time they waited to get pregnant. Therefore, a small number of children were karyotyped post-natally.[9] The percentage of patients who underwent prenatal diagnosis by chorionic villus sampling or amniocentesis was <50%, although all the patients were recommended to do these tests. In the case of a higher percentage of patients undergoing prenatal karyotyping, especially of the patients with an age of ≤34 years, the real figures of abnormal karyotypes will possibly be lower.

In all studies, there was a classification in epididymal sperm and testicular sperm, but testicular sperm was used in cases of OA and NOA. The study of Ludwig and Katalinic[13] had an apparently larger group of children conceived by ICSI using testicular sperm than children conceived by ICSI using epididymal sperm. It was not possible to discover in what cases testicular sperm was used and why there were more children after ICSI with testicular sperm. In the case of NOA (severe testicular failure), the spermatozoa are known to show a higher chromosomal aneuploidy rate.[22-25] The aneuploidy frequency in embryos obtained from NOA is also very high, but similar to embryos from OA.[26] Furthermore, it is assumed that genomic imprinting may be less complete when immature gametes are used.[27] However, all these concerns are theoretical, and the only study that compared the prevalence of congenital malformations in live born children obtained with testicular sperm of NOA men (n = 54) and children obtained with testicular sperm of OA men (n = 188) has not shown a difference.[28]

None of the studies made a difference in using fresh or frozen-thawed sperm or embryos. There was no difference between using fresh or frozen-thawed epididymal or testicular sperm on the outcome of ICSI, in spontaneous miscarriage rate, pregnancy and delivery rate.[29,30,31] No congenital anomalies were found; however, group sizes were too small to draw any valid conclusions. A recent study found a significantly higher major malformation rate in the ICSI cryo embryo live born group than in the fresh ICSI embryo live born group.[32] Other data suggest that malformation rates after cryopreservation seem to be comparable with those of fresh ICSI and fresh IVF.[33,34,35] More de novo karyotype anomalies were found prenatally in the cryo ICSI group compared with the fresh ICSI group, but this difference was not statistically significant.[32]

The total malformation rate (major + minor) has been shown to be higher in IVF/ICSI twins than in IVF/ICSI singletons, which is strongly associated with preterm birth of the twins.[36] Furthermore, the physical health of twins is poorer compared with singletons, because of prematurity and low birthweight;[37,38] therefore, especially in follow-up studies, it is important to know whether the children are singleton or a part of a multiple.

There are a number of limitations and sources of potential bias in this review; the studies described here, with the exception of Ludwig and Katalinic,[13] did not mention the stillborn and neonatal deaths and their cause of death and malformations, although this is important to know to draw valid conclusions. Thus, there might be an underestimation of the number of malformations. This is one of the possible biases in the studies. Secondly, the children born after IVF/ICSI are possibly more carefully investigated and malformations are more carefully recorded than in non-IVF/ICSI children; however, in none of the studies, blinding was applied, this will give another bias.

Not all major malformations are found at birth, but will be identified up to 12 months of age; two-thirds of major malformations were detected within the first 7 days of life and about 90% within the first 6 months.[39] Only in three of the studies, there was an investigation of the children at birth and at an older age; Bonduelle et al.[14] and Ludwig et al.[13] up to 2 months and Fedder et al.[16] up to 3 months–7 years (mean 20 months). It is therefore possible that in the other studies not all the malformations were identified within the study timeframe. Further, a proportion of children were lost to follow-up (Table I), because not all of them will come for examination or response the questionnaire, introducing further bias.

Although in the studies analysed a large number of ICSI children were included, most subgroups were small, even to detect a 2-fold increase (or decrease) in malformations. For example, if epididymal sperm retrievals account for only 5% of the ICSI children and if the prevalence of birth defects in the ejaculated ICSI group was 6%, you would need 168 children in the epididymal ICSI group in order to detect an RR of 2.0 and 431 to detect an RR of 0.5, with 80% power at 5% level of significance. On the basis of this example, only one-fifth epididymal sperm subgroups and two-fifths testicular sperm subgroups were large enough (n>168) to detect a 2-fold increase in birth defect risk, if such a large increase should exist. None of the subgroups was large enough (n >431) to detect a decreased risk of 0.5. In the Netherlands, 500 ICSI children were born between January 2002 and June 2009 after ICSI with epididymal sperm; only 35 children were born between March 2008 and June 2009 after ICSI with testicular sperm, because of a moratorium on this treatment until 2007 (unpublished data). This indicates that the subgroup of epididymal sperm is large enough to detect an RR of 0.5, but it will take over 5 years to reach such a large subgroup of testicular sperm.

Unfortunately, there was only one study about the physical, neurological and psychomotor development of children with a large lost for follow-up rate. So it is not possible to draw conclusions about this item.

Conclusions

Although there are no statistical differences in abnormal karyotypes, major malformations and follow-up of the children found in the studies we analysed, it should be considered that the study groups are small and heterogenic with numerous potential biases. We therefore call for standardized methodology for follow-up studies after ART, with a physical examination at birth and psychomotor assessment in childhood. Well-defined groups are necessary, such as ICSI with ejaculated sperm, ICSI with epididymal sperm and ICSI with testicular sperm and a control group of naturally conceived children. Maybe ESHRE could play a role in this process, by making standards for the methodology of follow-up of children after reproductive technologies.

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Chapter 9

Follow-up of children born after ICSI with epididymal spermatozoa

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Abstract

BACKGROUND: To evaluate the safety of ICSI with epididymal sperm, this study compared children born after ICSI treatment with epididymal sperm and children conceived after IVF and ICSI with ejaculated sperm. Additionally, the results of a multidisciplinary, multicentre follow-up of the children conceived with epididymal sperm at 2 years of age are described.

METHODS: This follow-up study included 378 children conceived after ICSI with epididymal sperm (percutaneous epididymal sperm aspiration: PESA group) and a control group of 1192 IVF and 1126 ICSI (with ejaculated sperm) children, all with a gestational age of 20 weeks or more. Questionnaires were sent at birth, 1 year and 4 years of age, collecting data on parental, pregnancy and child factors. A total of 148 PESA children were assessed at 2 years of age for motor performance, mental- and language development and compared with the Dutch norms.

RESULTS: PESA children showed no increased risks for stillbirths, total deaths and malformations. They also did not differ from IVF and ICSI children in gender rate, birthweight and gestational age. The mental Bayley score was higher ($P < 0.05$) for PESA singletons and parents reported fewer ($P < 0.05$) behavioural problems in the PESA group than the Dutch reference group. The scores for syntactic and lexical development for the PESA singletons were better ($P < 0.05$) than the Dutch standards.

CONCLUSIONS: ICSI with epididymal sperm does not lead to more stillbirths or congenital malformations in comparison to IVF and ICSI with ejaculated sperm and does not lead to poor development in comparison with the Dutch reference group.

Introduction

Azoospermia is present in ~5% of all infertile couples and is found in 10% of male infertility cases.[1,2] Since the introduction of ICSI in 1991, it is possible for these couples to father their own progeny by using sperm retrieved by percutaneous sperm aspiration (PESA), microsurgical epididymal sperm aspiration (MESA) or testicular sperm extraction (TESE). PESA and MESA are applied in cases of obstructive azoospermia (OA) with an assumed normal spermatogenesis. In cases of non-obstructive azoospermia (NOA), as the result of a testicular failure or in cases of unsuccessful PESA or MESA, TESE is indicated.

Introduction of ICSI with non-ejaculated sperm in the Netherlands has a special history. From 1994, ICSI in the Netherlands was carried out either with ejaculated semen or with non-ejaculated semen. However, a few years later concerns arose in the Dutch society about the health of the children born after ICSI with non-ejaculated sperm, referring to unknown risks of using aged (epidydimal) or immature (testicular) sperm cells. This led to a national moratorium for the application of ICSI with non-ejaculated sperm in 1996. With support from the Dutch government, studies were set up to obtain more information about these risks. Preclinical studies showed that motile epididymal sperm cells did not show increased DNA abnormalities, as measured by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling and chromomycin A3 assays.[3,4] Furthermore, studies in other countries showed no increase in the number of congenital defects in children born after ICSI in combination with MESA, PESA and TESE compared with ICSI with ejaculated sperm.[5,6,7] Therefore, the Dutch government agreed in 2001, with the start of a new prospective and multicentre clinical study, to confirm whether sperm retrieved by PESA or MESA in case of an OA can be used safely for ICSI. There was still a moratorium for the application of ICSI with TESE.

On the other hand, as recently published in a systematic review about karyotyping, congenital anomalies and follow-up of children after ICSI with non-ejaculated sperm, it was concluded that the study groups in these studies were small, heterogenic and with a lot of (possible) biases.[8] Therefore, well developed, large studies are necessary.

In this article, we will describe the children born during 7 years of ICSI treatment with epididymal sperm in the Netherlands. These results were compared with the results of children conceived after IVF and ICSI with ejaculated sperm. Additionally, we will describe the results of a multidisciplinary, multicentre follow-up of the children conceived with epididymal sperm at 2 years of age.

Materials and Methods

Setting

The protocol for this multicentre study was approved by the Dutch Central Committee on Research involving Human Subjects (CCMO, The Hague, The Netherlands). Patients from six IVF centres (the only ones in the Netherlands which may be performing PESA or MESA treatment before October 2007) were included: University Medical Centre St Radboud Nijmegen, University Medical Centre Utrecht, University Hospital Maastricht, University Medical Centre Rotterdam, VU University Medical Centre Amsterdam and Medical Centre for Childwish Leiderdorp.

Patients

All patients signed an informed consent for treatment and follow-up before participating in this study. Men with an azoospermia were seen by the urologist to diagnose whether there was an OA or NOA. In cases of an OA (anamnesis for obstruction, normal volume of testis, FSH <10 IU/l) a diagnostic PESA or MESA was planned to check if semen could be harvested. If possible this semen was cryopreserved for later treatment, otherwise an acute PESA/MESA was necessary at the time of ovum retrieval. Female patients were seen on the fertility department for a routine intake for an ICSI treatment; exclusion and inclusion criteria were according to the local clinical protocol. Included patients underwent ovarian hyperstimulation according to the local protocol. Only mature, morphologically normal oocytes were used for ICSI, either with frozen-thawed or fresh sperm cells. Intrauterine embryo replacement of one or two embryos was performed at 2–5 (dependent to the clinic) days after ovum retrieval.

Follow-up by questionnaires

The study group was called the 'PESA group', although in ~15% of the cases sperm was retrieved by MESA. The follow-up of these children was co-ordinated from Radboud University Nijmegen Medical Centre by asking the patients to return questionnaires after birth, after 1 year and 4 years of age. These questionnaires collected data on parental, pregnancy and child factors, including gestational age, mode of delivery, birthweight, presence or absence of malformations and neonatal problems. The study included all children with a gestational age of 20 weeks or more born between January 2002 and May 2008.

The control group consisted of IVF and ICSI (with ejaculated sperm) children with a gestational age of 20 weeks or more, who were born between June 1995 and May 2007 after treatment in the Radboud University Nijmegen Medical Centre. Information on fertility treatment and pregnancy outcome (birthweight, gender, alive or not at time of birth) of all children was retrieved from medical records (there is no national register for children born using IVF and ICSI). In addition, questionnaires were sent to the parents of all children near the first and fourth birthday to obtain data on parental, pregnancy and child factors. The questionnaire at 1 year of the control group obtained the same information as the combination of the questionnaires at birth and at 1 year of the PESA group.

If the parents (of the study group and control group) did not respond to the questionnaire, they received a reminder 3 months later. When they did not respond again at 1 year, the data asked in the 1-year questionnaire were included in the 4-years questionnaire. In both groups, only children born after replacement of fresh embryos were included. Only singletons and twins were included, because there were no triplets in the study group.

Follow-up by assessment

Additional to the questionnaires, the PESA group was assessed for motor performance, mental- and language development and compared with the Dutch norms according to the individual tests (explained below for the different tests). The PESA group was seen at chronological age of at least 2 years and 4 months at the department of Neonatology, Radboud University Nijmegen Medical Centre by a medical doctor, paediatric physical therapist, speech and language therapist and child psychologist during a single clinic visit. All observations were performed by one single person per area. Parents were present throughout the test procedures.

Physical examination was performed by a medical doctor, especially for validation of the questionnaires on major and minor malformations.

Motor performance and mental development was assessed using the Bayley Scales of Infant Development, 2nd edition, Dutch version (BSID-II-NL).[9,10] Based on content, construct and concurrent validity the BSID-II has been shown to be a comprehensive and appropriate instrument for assessing motor performance.[11,12] The results were compared with the Dutch standards as described by van der Meulen et al.,[10] which differ from the US norms at 2 years of age.[13] The BSID-II-NL motor scale assesses gross and fine motor skills. The mental scale consists of items that measure visual and auditive information processing, eye-hand coordination, imitation, language skills, memory and problem-solving. Raw scores are recalculated into a psychomotor developmental index (PDI) and a mental developmental index (MDI), with a mean value of 100 and a SD of 15 using norm-referenced tables. Scores of 115 or more are indicative of accelerated performance; scores between 114 and 85 reflect a normal performance. Scores between 84 and 70 reflect a mildly delayed performance and scores of 69 and less reflect a significantly delayed performance. Here, the classification 'accelerated' and 'normal' performances were combined into 'normal' performance.

Behavioural outcome was assessed using the Dutch version of the Child Behavior Checklist (CBCL) for children aged 1.5–5 years.[14] The CBCL is a validated parental questionnaire and rates 113 problem behaviour items on a 3-point scale ranging (0 = not true, 1 = somewhat true, 2 = very true). A total problem score is obtained by summing the scores of all items with a normative value of 50 and a SD of 10. Raw scores are recalculated into T scores. A T-score <60 is classified as normal, 60–63 is borderline and >63 is in the clinical range.

The speech therapist used the Dutch Reynell test, a standardized test that examines the receptive language development of Dutch-speaking children between 1.02 and 6.03 years of age.[15] In this test expressive language is not required since the children can point out their answers. To examine the language expression of the children, the tests for syntactic and lexical development of the Schlichting test were applied.[16] The Schlichting test is a standardized test for Dutch-speaking children of the same age group. The test for syntactic development elicited grammar structures and the test for lexical development measured the active vocabulary by instructing

the children to name objects and pictures. The numbers of correct answers resulting from both the Reynell and the Schlichting tests were transformed into aged-independent standard quotient scores: RLDQ = receptive language development quotient; SDQ = syntactic development quotient; LDQ = lexical development quotient. These scores had a mean of 100 and a SD of 15, with extreme values at −3 and + 3 SD.

Definitions

Except for late neonatal death (death of a child from day 7 until day 28 inclusive) and infant death (death of a child later than day 28),[7] all the definitions used conform with the glossary mentioned in 'The International Committee for Monitoring Assisted Reproductive Technology and the World Health Organization revised glossary on ART terminology, 2009'.[17] A widely accepted definition of major malformation was used, i.e. malformations that generally cause functional impairment or require surgical correction. The remaining malformations were considered minor. Ductus arteriosus was considered major if still patient at 3 months for a child born at term, or at 6 months for a preterm child born before 37 completed weeks. Inguinal hernia was considered minor in a preterm child born before 37 completed weeks and major for a child born after 37 weeks. Pyloric stenosis was counted as major.

Statistics

Descriptive statistics are given with mean and percentage with 95% confidence interval (CI). We used the independent samples t-test for comparing the means in birthweight between the PESA group and control groups. Descriptive statistics were used for the BSID-II-NL, classification and for the prevalence of language delay. The PDI, MDI, RLDQ, SDQ and LDQ were tested for normality according to the Dutch standards. One-sample t-tests were carried out to compare whether the children differed from the reference group. Independent sample t-tests were used to compare singletons and twins. P values <0.05 were considered statistically significant.

Results

Follow-up by questionnaires

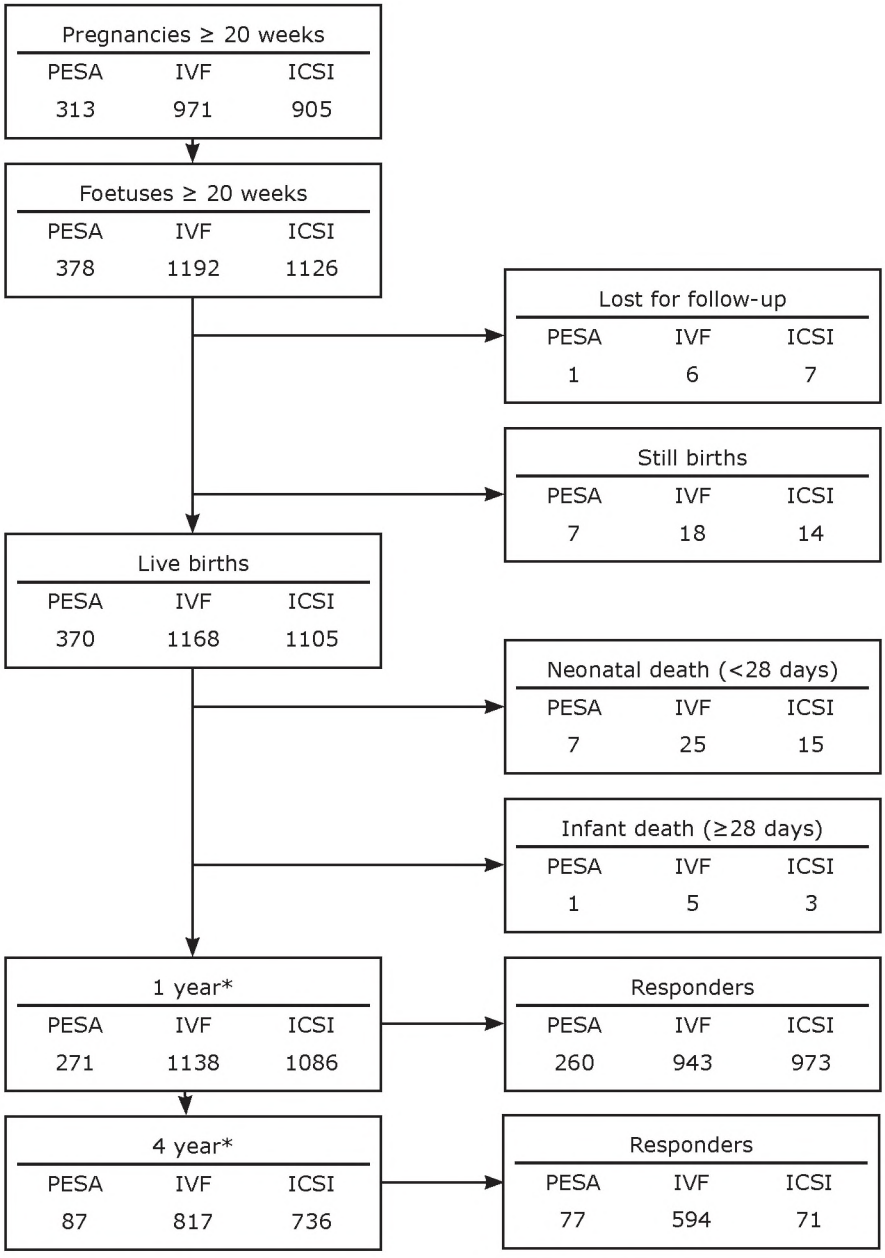
The flow chart (Figure 1) shows all included pregnancies with a gestational age of 20 weeks or more of the PESA, IVF and ICSI groups.

Table I shows details on stillbirths, live births and neonatal deaths of the fetuses at the age of 20 weeks or more, without the fetuses lost for follow-up. Of the stillbirths, six terminations (two PESA, three IVF and one ICSI) were performed after prenatal karyotyping. These terminations took place between 20 and 24 weeks of gestational age. Total stillbirths did not differ between groups. Also the causes for these deaths were comparable over the three groups, however only the IVF group showed perinatal loss resulting from solution placentae (n = 3). In general, most live born children who died before 1 year of age were related to twin pregnancies (40 out of 56). In total, 48 out of 56 children (85.7%) died after preterm birth (8 PESA, 26 IVF and 14 ICSI children) and of these 44 (78.6%) died after very preterm birth (7 PESA, 24 IVF and 13 ICSI children). For one IVF child who died the gestational age was not known. Major malformations as cause of death were present in one PESA child, seven IVF- and three ICSI children.

The first part of the follow-up of the children consisted of questionnaires. For the PESA group these were sent at birth, with a response of 99%. The response on the questionnaire at 1 year of age was 96% for the PESA group, 83% for the IVF group and 90% for the ICSI group. At 4 years of age the response was 89% for the PESA group, 73% for the IVF group and 75% for the ICSI group.

Table II shows the parental characteristics of the live born deliveries. In general the groups showed no differences. Exceptions are the lower proportion of primiparae in the IVF group and the relatively high age of fathers of the PESA children. Table III shows the paediatric characteristics of live born children. There was no difference in mean birthweight between the study group and the control groups, although there was a higher percentage of twins with a very low birthweight (<1500 g) in the PESA group than in the ICSI group. There were no differences between the groups in gender rate and gestational age.

Figure 1 Flow chart of all pregnancies ≥ 20 weeks with delivery between January 2002 and May 2008 for the PESA children and between June 1995 and May 2007 for the IVF and ICSI children



*reached the age of 1 year respectively 4 years before May 2008

Table I Live and stillbirths, neonatal and infant deaths (fetuses ≥ 20 weeks) following ICSI with epididymal sperm (PESA group) or IVF and ICSI using ejaculated sperm

	PESA			IVF			ICSI		
	Singletons	*Multiples*	*Total*	*Singletons*	*Multiples*	*Total*	*Singletons*	*Multiples*	*Total*
Total live births and stillbirths[a]	247	130	377	744	442	1186	679	440	1119
Stillbirths	4 (1.6%)	3 (2.3%)	7 (1.9%)	11 (1.5%)	7 (1.6%)	18 (1.5%)	10 (1.5%)	4 (0.9%)	14 (1.3%)
Live births	243	127	370	733	435	1168	669	436	1105
Early neonatal death (0 - 7 days)	2	2	4	5	17	22	4	9	13
Late neonatal death (7 - 28 days)	0	3	3	2	1	3	1	1	2
Infant death (28 days – 1 year)	0	1	1	1	4	5	1	2	3
Perinatal death[b]	6 (2.4%)	5 (3.8%)	11 (2.9%)	16 (2.2%)	24 (5.4%)	40 (3.4%)	14 (2.1%)	13 (3.0%)	27 (2.4%)
95% CI[c]	0.5-4.4	0.5-7.2	1.2-4.7	1.1-3.2	3.3-7.6	2.3-4.4	1.0-3.2	1.3-4.6	1.5-3.3
Total deaths	6 (2.4%)	9 (6.9%)	15 (4.0%)	19 (2.6%)	29 (6.6%)	48 (4.0%)	16 (2.4%)	16 (3.6%)	32 (2.9%)
95% CI[c]	0.5-4.4	2.5-11.4	2.0-6.0	1.4-3.7	4.2-8.9	2.9-5.2	1.2-3.5	1.9-5.4	1.9-3.9

[a] Without fetuses lost for follow up
[b] Stillbirths and early neonatal death together
[c] 95% confidence interval (CI) of percentage

The numbers of major and minor malformations of live born children are given in Table IV. There were no differences in major malformation rates between all groups. Minor malformations were seen less frequently in the PESA children than in the IVF and ICSI groups. There were no differences between singletons and twins in major or minor malformations.

Follow-up by assessment

The characteristics of the parents of the assessed group (i.e. who did follow-up) were comparable with the total PESA group (Table V). Table VI shows the results of the follow-up scores of the PESA children at the age of at least 2 years and 4 months. Of 167 invited children, 4 children moved with unknown address and 14 children (6 singletons and 4 twins) did not appear for different reasons such as follow-up programme elsewhere, operation or illness, forgotten or did not want to come. In total, there were 149 children assessed. Reliable assessment was not possible in all the children, because some refused items or did not understand the instructions owing to language barriers (9 children had another language, although one or both parents understood Dutch).

No additional malformations were found by physical examination than already found and reported at 1 year of age.

The BSID-II-NL showed no differences for the mean PDI (motor performance) between the study group and the Dutch reference group.

The mean MDI (mental development) of the PESA group was within the normal range but was higher ($P < 0.05$) than the Dutch reference group. This positive effect was only present in the singleton subgroup; the twin subgroup did not differ from the norm group. There were more than the expected numbers of PESA children with normal mental development and fewer PESA children at risk or abnormal.

Concerning the CBCL, parents reported fewer behavioural problems than the norm. In the overall group the behavioural scores were lower ($P < 0.05$) than the normative mean value of 50 +SD (10). No differences were found when comparing the singletons and twins.

Table II Parental characteristics of those with a live birth(s)

	PESA		IVF		ICSI	
	n/mean	*%;95%CI/range (SD)*	*n/mean*	*%;95%CI/range (SD)*	*n/mean*	*%;95%CI/range (SD)*
Total deliveries	308		952		888	
Singletons	243	78.9; 74.2-83.5	733	77.0; 74.3-79.7	669	75.3;72.4-78.2
Twins	65	21.1; 16.5-25.8	219	23.0; 20.3-25.7	219	24.7;21.8-27.6
Maternal age (years)[a]						
Total	33.4	23.1-41.9 (3.7)	34.3	21.5-43.5 (4.0)	33.4	20.7-42.7 (3.8)
Singletons	33.5	23.1-41.9 (3.7)	34.6	21.5-43.5 (4.0)	33.6	21.3-42.7 (3.8)
Twins	33.1	23.6-40.8 (3.6)	33.5	21.5-41.0 (3.8)	32.9	20.7-41.2 (3.7)
Paternal age (years)[a]						
Total	41.1	26.8-67.2 (7.9)	36.7	21.4-61.4 (4.9)	36.5	22.7-56.8 (5.2)
Singletons	41.4	26.8-64.2 (8.1)	36.9	21.5-61.4 (4.9)	36.6	22.7-56.8 (5.3)
Twins	40.0	29.8-67.2 (7.2)	36.1	21.4-58.9 (5.1)	36.1	25.6-55.0 (4.9)
Maternal smoking[b]						
No smoking	284	93.4; 90.6-96.3	714	90.2; 88.0-92.2	720	91.5; 89.5-93.5
Smoking	20	6.6; 3.7-9.4	78	9.8; 7.7-12.0	67	8.5; 6.5-10.5
Unknown	4		160		101	
Education mother						
Low[c]	46	15.2; 11.1-19.3	125	15.8; 13.2-18.4	123	15.6; 13.0-18.2
High[d]	257	84.8; 80.7-88.9	667	84.2; 81.6-86.8	665	84.4; 81.8-87.0
Unknown	5		160		100	

Table II Continued

	PESA		IVF		ICSI	
	n/mean	*%;95%CI/range (SD)*	*n/mean*	*%;95%CI/range (SD)*	*n/mean*	*%;95%CI/range (SD)*
Education father						
Low[c]	48	15.9; 11.7-20.1	172	21.8; 18.9-24.8	159	20.2; 17.3-23.1
High[d]	254	84.1; 79.9-88.3	616	78.2; 75.2-81.1	628	79.8; 76.9-82.7
Unknown	6		164		101	
Parity						
First	243	79.9; 75.3-84.5	546	69.2; 65.9-72.5	599	75.7; 72.7-78.8
≥ Second	61	20.1; 15.5-24.7	243	30.8; 27.5-34.1	192	24.3; 21.2-27.3
Unknown	4		163		97	

[a] at time of delivery
[b] during pregnancy
[c] low: (no) primary school or lower level of secondary school and vocational training
[d] high: medium and higher level of secondary school or medium and higher level of vocational training or university

Table III Paediatric characteristics of live births

	PESA		IVF		ICSI	
	n/mean	*%;95%CI/range (SD)*	*n/mean*	*%;95%CI/range (SD)*	*n/mean*	*%;95%CI/range (SD)*
Total	370		1168		1105	
Singletons	243	65.7; 60.7-70.6	733	62.8; 59.5-65.6	669	60.5; 57.6-63.5
Twins	127	34.3; 29.4-39.3	435	37.2; 34.4-40.1	436	39.5; 36.5-42.4
Gender						
Boys	181	49.6; 44.4-54.8	565	51.9; 48.9-54.9	504	48.0; 44.9-51.0
Girls	184	50.4; 45.2-55.6	524	48.1; 45.1-51.1	547	52.0; 49.0-55.1
Unknown	5		79		54	
Birthweight (gram)						
Total	2983.2	500-4840 (624.9)	2932.5	375-4885 (776.6)	2967.6	350-5180 (748.5)
Singletons	3315.6	500-4840 (655.6)	3271.5	375-4885 (613.7)	3318.2	720-5180 (580.2)
Twins	2337.1	765-4010 (733.4)	2359.3	385-4055 (665.0)	2434.5	350-4288 (616.1)
Unknown	2		19		6	
Birthweight < 1500 g						
Total	23	6.3; 3.7-8.8	42	3.7; 2.5-4.8	28	2.5; 1.6-3.5
Singletons	3	1.2; 0.0-2.7	8	1.1; 0.3-1.9	3	0.5; 0.0-1.0
Twins	20	16.0; 9.4-22.6	34	8.0; 5.3-10.6	25	5.7; 3.5-8.0
Birthweight < 2500 g						
Total	91	24.7; 20.2-29.2	222	19.3; 17.0-21.7	225	20.5; 18.0-22.9
Singletons	23	9.5; 5.7-13.2	43	6.0; 4.2-7.7	41	6.2; 4.3-8.1
Twins	68	54.4; 45.5-63.2	179	41.9; 37.1-46.7	184	42.2; 37.5-46.9

Table III Continued

	PESA		IVF		ICSI	
	n/mean	*%;95%CI/range (SD)*	*n/mean*	*%;95%CI/range (SD)*	*n/mean*	*%;95%CI/range (SD)*
Gestational age (weeks)						
Total	38.0	23.3-42.4 (3.3)	38.0	21.9-43.1 (3.3)	38.2	20.4-43.3 (3.0)
Singletons	39.3	23.3-42.4 (2.4)	39.3	24.6-43.1 (2.4)	39.4	20.9-43.3 (2.2)
Twins	35.6	27.1-42.1 (3.4)	35.7	21.9-40.0 (3.6)	36.3	20.4-40.6 (3.1)
Unknown	6		133		71	
Prematurity < 37 weeks						
Total	84	23.1; 18.7-27.5	262	25.3; 22.6-28.0	227	22.0; 19.4-24.5
Singletons	22	9.1; 5.4-12.8	70	10.6; 8.2-12.9	46	7.3; 5.2-9.4
Twins	62	50.4; 41.4-59.4	192	51.6; 46.4-56.8	180	44.3; 39.4-49.3

Table IV Major and minor malformations of live born children

	PESA		IVF		ICSI	
	n	*%;95%CI*	*n*	*%;95%CI*	*n*	*%;95%CI*
Total	370		1168		1105	
Major malformation[a]	13	3.6; 1.6-5.5	46	4.8; 3.4-6.2	33	3.4; 2.2-4.6
Minor malformation[b]	27	7.4; 4.7-10.1	142	14.9; 12.6-17.2	147	15.2; 12.9-17.5
No malformations	325	89.0; 85.8-92.3	766	80.3; 77.7-82.9	788	81.4; 78.9-83.9
Unknown	5		214		137	
Singletons	243		733		669	
Major malformation[a]	8	3.3; 1.0-5.6	28	4.5; 2.8-6.1	18	3.0; 1.6-4.4
Minor malformation[b]	17	7.0; 3.7-10.3	92	14.6; 11.8-17.4	100	16.7; 13.6-19.7
No malformations	217	89.7; 85.8-93.6	509	80.9; 77.7-84.1	481	80.3; 77.1-83.6
Unknown	1		104		70	
Twins	127		435		436	
Major malformation[a]	5	4.1; 0.5-7.6	18	5.5; 3.0-8.1	15	4.1; 2.0-6.1
Minor malformation[b]	10	8.1; 3.2-13.1	50	15.4; 11.4-19.4	47	12.7; 9.3-16.2
No malformations	108	87.8; 81.9-93.7	257	79.1; 74.6-83.6	307	83.2; 79.3-87.1
Unknown	4		110		67	

A widely accepted definition of major malformation was used i.e. malformations that generally cause functional impairment or require surgical correction. The remaining malformations were considered minor. Ductus arteriosus was considered major if still patent at 3 months for a child born at term, or at 6 months for a preterm child born before 37 completed weeks. Inguinal hernia was considered minor in a preterm child born before 37 completed weeks and major for a child born after 37 weeks. Pyloric stenosis was counted as major.

[a] number of children with one or more major malformations or major and minor malformations

[b] number of children with one or more minor malformations and no major malformations

Discussion

In this prospective multicentre study, it was found that children born after an ICSI treatment with epididymal sperm of men with an OA showed no increased risks for stillbirths, total deaths, malformations and psycho-motor problems. They also did not differ from IVF and ICSI (with ejaculated sperm) children in gender rate, birthweight and gestational age. On some aspects the PESA group scored even better than the IVF and ICSI control groups. The minor malformations rate in the PESA group was lower than in the control groups. Furthermore, the mental Bayley score was significantly higher for PESA singletons, and parents reported fewer behavioural problems in the PESA group than the Dutch reference group. Finally, the scores for syntactic development and lexical development for the PESA singletons were significantly better than the Dutch standards.

The percentage of stillbirths was in accordance with other studies for IVF and ICSI children, although these percentages are higher than for children conceived naturally.[7,18,19,20] It is thought that infertility, irrespective of treatment, increases the risk of perinatal death and other problems in the offspring.[21-25] A higher incidence of low birthweight, preterm birth and perinatal death is not only a complication of multiple pregnancies but is also more prevalent in IVF and ICSI singletons.[19] The actual infertility is a possible explanation for this phenomenon but more obvious is the fact that the women who conceive after IVF or ICSI are often older and primiparous. Also vanishing twins are seen more often in women who conceive after IVF or ICSI and may cause lower birthweight, preterm birth and perinatal death.[26,27,28]

In our study, there were no differences in gender rate between groups. However, there were slightly more girls in the PESA group and ICSI group (with ejaculated sperm) in contrast to slightly more boys in the IVF group. In other studies they also found more boys after an IVF treatment, but different results for sex ratios after ICSI with ejaculated or epididymal sperm. Bonduelle et al.[7] found more boys after ICSI with epididymal sperm and Fedder et al.[29] found more girls, although both results were not significant and were based on small numbers in groups only.

In agreement with the present results, previous studies showed no increased risks related to the use of epididymal sperm.[7,30] In different meta-analyses, a higher rate of malformations has been reported for

Table V Parental and Paediatric characteristics of the assessed PESA children at 2 years of age and total PESA group

	Total PESA group		Group of assessed PESA children	
	n/mean	*%;95%CI/range*	*n/mean*	*%;95%CI/range*
Total	370		149	
Singletons	243	65.7; 60.7-70.6	92	61.7; 53.8-69.7
Twins	127	34.3; 29.4-39.3	57	38.3; 30.3-46.2
Maternal age (years)[a]				
Total	33.4	23.1-41.9	33.2	23.1-40.8
Paternal age (years)[a]				
Total	41.1	26.8-67.2	40.6	26.8-61.3
Education mother				
Low[b]	46	15.2; 11.1-19.3	17	14.0; 7.7-20.4
High[c]	257	84.8; 80.7-88.9	104	86.0; 79.6-92.3
Unknown	5		1	
Education father				
Low[b]	48	15.9; 11.7-20.1	20	16.5; 9.7-23.3
High[c]	254	84.1; 79.9-88.3	101	83.5; 76.7-90.2
Unknown	6		1	
Gender				
Boys	181	49.6; 44.4-54.8	70	47.0; 38.8-55.1
Girls	184	50.4; 45.2-55.6	79	53.0; 44.8-61.2
Unknown	5			

Table V Continued

	Total PESA group		Group of assessed PESA children	
	n/mean	*%;95%CI/range*	*n/mean*	*%;95%CI/range*
Birth weight (gram)				
Total	2983.2	500-4840	2966	1080-4440
Singletons	3315.6	500-4840	3358	1625-4440
Twins	2337.1	765-4010	2334	1080-3400
Unknown	2			
Gestational age (weeks)				
Total	38.0	23.3-42.4	38.2	29.6-42.4
Singletons	39.3	23.3-42.4	39.6	33.3-42.4
Twins	35.6	27.1-42.1	36.0	29.6-42.1
Unknown	6			
Prematurity < 37 weeks				
Total	84	23.1; 18.7-27.5	39	26.2; 19.0-33.4
Singletons	22	9.1; 5.4-12.8	7	7.6; 2.1-13.1
Twins	62	50.4; 41.4-59.4	32	56.1; 43.0-69.3

[a] at time of delivery
[b] low: (no) primary school or lower level of secondary school and vocational training
[c] high: medium and higher level of secondary school or medium and higher level of vocational training or university

Table VI Follow-up outcome of the PESA children at 2 years of age

	Total		
	n (%)/mean (SD)		
Bayley score: motor			
Normal (> -1 SD)	105 (75.0)		
At Risk (-2 < x < -1 SD)	20 (14.3)		
Abnormal (< -2 SD)	15 (10.7)		
Missing	9		
PDI: mean (SD)	97.6 (21.2)		
Student-t test	-1.37, p = 0.173		
Bayley score: mental			
Normal (> -1 SD)	132 (95.7)		
At Risk (-2 < x < -1 SD)	4 (2.9)		
Abnormal (< -2 SD)	2 (1.4)		
Missing	11		
MDI: mean (SD)	103.8 (11.6)		
Student-t test	3.811, p < 0.05		
CBCL: total problem score			
Normal range (< 60)	123 (93.9)		
Borderline (60-63)	2 (1.5)		
Clinical range (>63)	6 (4.6)		
Missing	18		
CBCL: mean (SD)	46.9 (9.5)		
Student t-test	-3.642, p < 0.05		
Receptive language development (Reynell test)			
Normal (> -1 SD)	132 (94.3)		
At Risk (-2 < x < -1 SD)	8 (5.7)		
Abnormal (< -2 SD)	-		
Missing	9		
RLDQ: mean (SD)	100.3 (SD 9.27)		
Student t-test	0.364, p = 0.717		

	Singletons	Twins
	n (%)/mean (SD)	*n (%)/mean (SD)*
	70 (77.8)	35 (70.0)
	14 (15.5)	6 (12.0)
	6 (6.7)	9 (18.0)
	2	7
	99.2 (20.8)	94.7 (21.7)
	-0.385, p = 0.701	-1,742, p =0.088
	82 (96.5)	50 (94.3)
	3 (3.5)	1 (1.9)
	0	2 (3.8)
	7	4
	105.1 (11.1)	101.6 (12.1)
	4.271, p < 0.05	0.930, p = 0.356
	73 (93.6)	50 (94.3)
	1 (1.3)	1 (1.9)
	4 (5.1)	2 (3.8)
	14	4
	47.69 (9.3)	45.94 (9.7)
	-2.196, p < 0.05	-3.030, p < 0.05
	81 (93.1)	51 (96.2)
	6 (6.9)	2 (3.8)
	-	-
	5	4
	101.2 (SD 9.91)	98.7 (SD 8.04)
	1.158, p = 0.250	-1.145, p = 0.258

Table VI Continued

	Total		
	n (%)/mean (SD)		
Syntactic development (Schlichting test)			
Normal (> -1 SD)	128 (94.2)		
At Risk (-2 < x < -1 SD)	8 (5.8)		
Abnormal (< -2 SD)	-		
Missing	13		
SDQ: mean (SD)	101.7 (SD 10.22)		
Student t-test	1.970, p = 0.051		
Lexical development (Schlichting test)			
Normal (> -1 SD)	118 (92.9)		
At Risk (-2 < x < -1 SD)	9 (7.1)		
Abnormal (< -2 SD)	-		
Missing	22		
LDQ: mean (SD)	100.9 (SD 11.09)		
Student t-test	0.888, p = 0.376		

Bayley, Reynell and Schliching tests: the normal group has a mean ± SD of 100 ± 15.
CBCL (=Child Behaviour Checklist): the normal group has a normal mean ± SD of 50 ± 10.
Student's t-test was performed over the means, SD's and numbers of the study group vs the Dutch reference group.
PDI = Psychomotor Developmental Index; MDI = Mental Developmental Index; RLDQ = Receptive language development quotient; SDQ = Syntactic development quotient; LDQ = Lexical development quotient

	Singletons	Twins
	n (%)/mean (SD)	*n (%)/mean (SD)*
	82 (96.5)	46 (90.2)
	3 (3.5)	5 (9.8)
	-	-
	7	6
	103.7 (SD 9.44)	98.1 (SD 10.68)
	3.654, p < 0.05	-1.259, p = 0.214
	72 (93.5)	46 (92.0)
	5 (6.5)	4 (8.0)
	-	-
	15	7
	103.0 (SD 11.65)	97.6 (SD 9.47)
	2.247, p <0.05	-1.762, p = 0.084

For speech and language development (Reynell test and Schlichting tests), the PESA singleton group showed a better ($P < 0.05$) mean score for syntactic and lexical development (SDQ and LDQ) as compared with the Dutch reference values. The receptive language development (Reynell test) showed no differences for the mean RDLQ between the study group and the Dutch reference group.

IVF and ICSI children than children conceived naturally but these rates did not differ between IVF and ICSI.[31-34] Moreover, the risk of major malformations did not seem to be related to the source of sperm.[8] In general, various methodological pitfalls were mentioned in studies about malformations, such as failure to take into account potential confounding variables, the way in which malformations are determined and registered, and inconsistent criteria with respect to classifying malformations (inclusion or exclusion of minor malformations and malformations present in terminated pregnancies and stillbirths).[35,36] Because of these pitfalls and because of studies with only small groups of children conceived after ICSI with epididymal sperm, it was difficult to compare the finding of fewer minor malformations in our PESA group. Not all malformations are diagnosed at birth but will be identified up to 12 months of age; two-thirds of major malformations were detected within the first 7 days of life and about 90% within the first 6 months.[37] The data on malformations of the IVF and ICSI children were all from the questionnaires for 1 year old. As only 260 out of 357 (72.8%) live born PESA children reached the age of 1 year before May 2008, this could partly explain the lower prevalence of minor malformations in this group.

In one of the first studies about development of the ICSI children, a lower mental development with Bayleys at 1 year of age was found, especially for boys.[38] These findings were not confirmed by later studies, which showed no significant difference in development between the ICSI children and their naturally conceived peers.[38,39,40] Studies about children conceived after ICSI with epididymal sperm with a follow-up of at least 2 years are rare.[8,41] Our findings of a significantly higher mental Bayley score for PESA singletons and fewer reported behavioural problems in the PESA group than the Dutch reference group could be explained by a higher educational level of the parents of the PESA children than of the Dutch reference group [high education of mother in 77.0% and of father in 73.1%[10]]. The same explanation could be given for the significantly better scores for syntactic development and lexical development for the

PESA singletons than the Dutch standards.

The strengths of our study were the large numbers in the study and control groups, the similar way of collecting information for all groups using the same questionnaires with a good response, the availability of information about the parents and children, and the long period of follow-up of the children up to 4 years of age. Possible weaknesses of our study were the composition of the control group (no natural conceived children but IVF and ICSI children), the use of questionnaires, the un-blinded design and the lack of matched controls for the follow-up at 2 years of age.

The reassuring outcomes of the present study are important not only for clinicians and future patients but also for policy-makers who have to decide about ethics and costs of new techniques. At time of writing, the questionnaires (at birth, 1 year and 4 years of age) have been sent to the parents of the PESA children conceived in Nijmegen. We stopped the assessment at 2 years of age. The first 90 PESA children seen at 2 years of age by assessment were already seen at 5 years of age for another assessment by a medical doctor, a psychologist and a physical therapist, and unfortunately our project has been stopped for reasons of cost effectiveness, therefore no additional data at 5 years will be collected. Our study focused on ICSI with epididymal sperm and not on ICSI with testicular sperm. More research is necessary to confirm whether testicular sperm retrieved by TESE in case of an NOA can be used safely for ICSI.

In conclusion, ICSI with epididymal sperm does not lead to more stillbirths and congenital malformations in comparison with IVF and ICSI with ejaculated sperm and does not lead to poor development in comparison with the Dutch reference group.

Authors' roles

G.H.W. and J.A.M.K had the idea and designed this manuscript. G.H.W, M.H, A.J.W.M.J, J.J.C.M.R, and S.A.F.G did the acquisition and analysis of the data. G.H.W. was the main author. All authors revised the article critically.

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Discussion and summary

Chapter 10

General discussion

The main aim of this thesis was to evaluate the health, growth and development of children conceived after ICSI with ejaculated and especially with non-ejaculated sperm.

Firstly, the theoretical risks and complications for women, men and children were described in this thesis after a literature search. Secondly, a systematic review was carried out to investigate the karyotypes of foetuses, congenital anomalies and the follow-up of the children born after ICSI with epididymal or testicular sperm. Thirdly, an explorative study was described in order to identify constitutional de novo DNA copy number changes, including those that are phenotypically silent. Fourth, a questionnaire was sent to parents of IVF and ICSI children at one and four years of age. This provided us with data about the parents, pregnancy (e.g. pre-eclampsia) and child (e.g. growth). Finally, a clinical study was carried out with the follow-up of children conceived after ICSI with epididymal sperm.

In this chapter the answers to the main questions are mentioned and discussed. The most relevant methodological issues are reviewed and at the end the implications for the different stakeholders and recommendations for future research are given.

Answers to the main questions

1. Theoretical risks of ICSI (especially with non-ejaculated sperm) may result in fertilisation failure, miscarriages, birth defects, genetic abnormalities, developmental abnormalities and infertility in the offspring. **(Chapter 2)**

2. Diminished responsiveness of the ovaries to FSH stimulation in an IVF cycle, reflecting decreased ovarian reserve, is associated with an increased risk of developing pre-eclampsia in a subsequent pregnancy. **(Chapter 3)**

3. Although IVF and ICSI singletons had a statistically significantly lower birth weight than naturally conceived singletons, the average individual weight curves showed that this difference disappeared before the age of 4 years. **(Chapter 4)**

4. ICSI with epididymal sperm was successfully used as a treatment of infertility caused by obstructive azoospermia in the first year after restarting this treatment under conditions in the Netherlands. **(Chapter 5 and 6)**

5. ICSI children had more de novo 'same direction genomic copy number changes' than the control group of naturally conceived children. **(Chapter 7)**

6. A systematic review of studies on the health of children born after ICSI with ejaculated, epididymal or testicular sperm showed no statistical differences between the study groups in karyotypes (two studies), malformations (five studies) and follow-up (one study). **(Chapter 8)**

7. ICSI with epididymal sperm does not lead to more stillbirths or congenital malformations in comparison with IVF and ICSI with ejaculated sperm and does not lead to poor development at two years of age in comparison with the Dutch reference group. **(Chapter 9)**

Interpretation of the answers

There are theoretical risks of ICSI that may result in fertilisation failure, abortion, birth defects, genetic abnormalities, developmental abnormalities and infertility in the offspring. Surgical retrieved sperm from the epididymis or from the testis are of special concern, because of additional risks such as aging due to a prolonged stay in the epididymis (in case of obstruction) and incomplete maturation (in cases of non-obstructive azoospermia).[1] This is important to know for counselling the future parents, especially if these risks will result in genetic abnormalities and birth defects. The cause of infertility is also of concern, because these risks may be due to the infertility itself, rather than the ICSI technique.[2] Moreover, a causal relationship between ICSI and adverse effects on the offspring is difficult to make, because in most studies maternal characteristics (such as age and parity), co morbidity, life-style (smoking, drinking, drugs) and the fact that ICSI children are more often born prematurely (singletons and especially multiples), with a low birth weight are confounding factors.[3]

Beside possible effects of ICSI treatment for the children, there are possible short-term complications for women after assisted reproductive technology such as pre-eclampsia.[4,5] Pre-eclampsia affects 2 -10% of all pregnancies and relates to substantial maternal and fetal morbidity

and mortality.[6,7] In our study we explored a possible association between ovarian reserve, as measured by basal ovarian function and response capacity to FSH stimulation on one hand and the development of pre-eclampsia as pregnancy-related vascular complication on the other. The administered FSH per follicle and FSH per obtained oocyte was the most powerful predictor of all variables studied. In accordance to other studies we observed a shorter pregnancy duration in the pre-eclampsia subgroup, which was expected considering the tendency to induce delivery in case of preeclampsia. Although these children had a lower birth weight, after adjustment for gestational age, parity and sex, the outcome of the children born after pre-eclampsia and the children in the control group were comparable. This is in contrast with findings of several other studies that state that birth weight even after adjustment for gestational age is lower in children born to mothers with pre-eclampsia.[8] Possibly we did not observe this in our study because of the limited number of cases.

Although the indication for ICSI treatment is based on the male subfertility, it is important to have a partner with a sufficient ovarian reserve because of the lower pregnancy rate, the higher risk on pre-eclampsia and possible effects on birth weight and fetal morbidity in women with a low ovarian reserve. This knowledge is important, because it is already known from other studies, that IVF and ICSI singletons had a significantly lower birth weight than reference singletons.[9] In our study we confirmed that term IVF and ICSI singletons had a significantly lower birth weight than children from a national reference group. The mechanism behind this difference in birth weight is unknown, but several explanations have been given, such as a late effect of the hormonal ovary stimulation, the IVF/ICSI procedure itself (oocyte retrieval, culture medium, embryo transfer), the pre-existing condition of the mother, factors related to subfertility or double-embryo transfer.[10,11,12] It was not possible in our study to verify all these explanations. There were no differences in birth weight between the IVF and ICSI group, so it is not likely that the factors related to subfertility or the ICSI procedure itself are of a great influence. In most cases the indication for ICSI was only male related, most women did not have a fertility problem.

In this thesis we were interested in the health of the ICSI children, and growth is an important aspect of health in children. Especially because reduced birth weight and delayed growth are not without health consequences, like cardiovascular disease in later life.[13,14] Some studies

reported already on postnatal growth of IVF children[15-20] and other studies about ICSI children as well.[21-23] They have some limitations such as having not differentiated for gestational age or performed only one single weight measurement at five years of age. Our study differentiated for gestational age, had at least four measurements and included both IVF and ICSI children. It showed that the longitudinal growth of IVF and ICSI children is comparable with the growth of natural conceived children and that the difference in birth weight disappeared before the age of four years.

In this thesis we were especially interested in children conceived after ICSI with non-ejaculated sperm. In the Netherlands, in 2001 a prospective and multicentre clinical study was started to confirm whether sperm retrieved by PESA or MESA in cases of an obstructive azoospermia, can be used safely for ICSI. Evaluation after one year showed that ICSI with sperm retrieved by PESA was an effective treatment for couples with childlessness due to an obstructive azoospermia. Beside this, no congenital defects were found in any of the seventeen children, born in this first year.

In one pregnancy, a chromosomal anomaly was diagnosed by amniocentesis, probably a de novo anomaly. It is not clear whether this diagnosis was based on coincidence, or whether it was due to the ICSI-treatment or to the PESA-procedure. However, a significantly higher percentage of de novo chromosomal anomalies has been observed in ICSI-mediated offspring,[24] indicating that it remains necessary to pay attention to this aspect.

This significantly higher rate of de novo chromosomal anomalies suggests that ART procedures increase the genetic load. Such an increase, if present, could besides gross chromosomal changes including copy number variation of large kilobase (kb) range sequences, also apply to other mutation types such as simple sequence repeat instability, small deletions, duplications, inversions and base pair changes. It is not known to what extent an increased genetic load might manifest itself in congenital malformations, so we initiate an explorative study in order to identify constitutional de novo DNA copy number changes. Significantly more 'same direction copy number changes' (i.e. simultaneous copy number gain [or loss] with respect to both parents) were found in ICSI children compared to children from the control group. No significant correlation was found for 'same direction copy number changes' in children born after ICSI with sperm from a PESA and in children born after ICSI with ejaculated sperm from

fathers suffering extreme OAT. None of the children had major or minor malformations, so the genomic changes do not seem to have phenotypic consequences.

Beside our own first observations about the health of children born after ICSI with epididymal sperm, we were interested in the literature about abnormal karyotypes, congenital anomalies and the follow-up of the children born after ICSI with non-ejaculated sperm. The studies found in the systematic review suggest that the risk ratios may be similar in the subgroups of the ICSI procedures (with epididymal sperm, testicular sperm or ejaculated sperm). Points of attention are the possible heterogeneity and the low number of children in some subgroups resulting in large confidence intervals (CI's). Possible heterogeneity between the study groups in the articles, are related to the age of the mother, indication for PESA or TESE, difference in OA and NOA, the use of fresh or frozen thawed sperm or embryos and difference in percentage of singletons and multiples.

In the Netherlands there was a national moratorium for the application of ICSI with non-ejaculated sperm from 1996 until 2001. Studies in other countries showed no increase in the number of congenital defects in children born after ICSI in combination with MESA or PESA compared to ICSI with ejaculated sperm,[25,26,27] so the Dutch government agreed in 2001 to start a prospective and multicentre clinical study to confirm these findings. In this study it was found that children born after an ICSI treatment with epididymal sperm of men with an obstructive azoospermia showed no increased risks for stillbirths, total deaths, malformations and psycho motor problems. In different meta-analysis a higher rate of malformations has been reported for IVF and ICSI children compared to natural conceived children, but these rates did not differ between IVF and ICSI.[28-31] In our study group (ICSI with epididymal sperm) we found the same rate of major malformations and a lower rate of minor malformations than in the IVF and ICSI (with ejaculated sperm) groups. Furthermore, the mental Bayley score, the scores for syntactic development and lexical development were significantly higher in singletons of the study group and parents reported less behavioural problems in this group than the Dutch reference group.

Methodological issues

The two reviews (chapter 2 and chapter 7) mentioned some methodological issues of the reviewed studies, like differences in study design and study groups. Most studies were cohort or case control studies, none of them were matched, some were retrospective, by identifying health registers and some were prospective, by physical examination or by physical examination as well as by sending questionnaires. Rarely a study made adjustment for some variables, which could act as confounders: year of birth, maternal age and parity, years of involuntary childlessness and maternal smoking in early pregnancy. Possible heterogeneity between the study groups in the articles, are related to the age of the mother, in- or exclusion of stillbirths, indication for PESA or TESE, difference in OA and NOA, the use of fresh or frozen thawed sperm or embryos and difference in percentage of singletons and multiples. Beside this possible heterogeneity there were low numbers of children in some subgroups resulting in large CI's.

The selection of cases in the case-control study about risk of developing pre-eclampsia, was based on self report of the women in the questionnaires. Although all data were verified in both cases and controls, the reported incidence of preeclampsia was low compared to that of the general population, especially since preeclampsia is stated to occur more frequently in pregnancies resulting from ART.[5] These figures could also be biased by our local selection protocol prior to IVF, which excluded women having a BMI above 32 kg/m^2. Overweight increases the chance of pregnancy induced hypertension.[32] Beside this, the low number of women reporting preeclampsia resulted in a small case group that led to a modest power, although the strength of the study was the design as case-control study.

In the explorative study to identify constitutional de novo DNA copy number changes, we used Bacterial Artificial Chromosome (BAC)-array mediated comparative genomic hybridization (array CGH) analysis. The tiling-resolution BAC arrays used provide unbiased genomic coverage, including polymorphic genomic intervals at a practical resolution of around 100 kb. Although executed at a small scale, our search revealed a relative abundance of 'same direction copy number changes', but a larger group is needed to confirm these findings.

The study about weight and growth of the IVF and ICSI children had a large sample size (especially the reference group), high response rate on the questionnaires and relatively long follow-up with at least four measurements up to four years of age. A disadvantage was the comparison between longitudinal data of IVF and ICSI children with cross-sectional data of a reference group, although it gave us the opportunity to have a large population based control group of natural conceived children.

The last clinical study about the follow up of the children born after ICSI with epididymal sperm had well defined groups of children conceived after IVF, ICSI with ejaculated sperm or ICSI with epididymal sperm. Other strengths of the study were the large numbers in the study and control groups, the similar way of collecting information of all groups by the same questionnaires with a good response, the availability of information about the parents and children and the long period of follow-up of the children up to four years of age. Possible weaknesses of this study were the composition of the control group (no natural conceived children but IVF and ICSI children), the use of questionnaires, the un-blinded design and the lack of matched controls for the follow-up at two years of age.

Implications for stakeholders

The reassuring outcomes of these studies are important for clinicians and future patients, but also for policymakers who have to decide about ethics and costs of new techniques.

Although some concerns about the findings of lower birth weight of IVF and ICSI children compared to naturally conceived singletons remain, these differences disappeared before the age of 4 years. There were statistical significant more 'same direction copy number changes' of DNA in ICSI children (with ejaculated and epididymal sperm) compared to in the control group, but there were not more congenital malformations found in ICSI children conceived with ejaculated or epididymal sperm compared to IVF children. In the literature and in our clinical follow up study, there were not more concerns about children born after ICSI with epididymal sperm compared to ICSI with ejaculated sperm or after IVF. There are still concerns about the mortality and morbidity of multiples, which are seen more often after IVF and all ICSI treatment.

The future patients have to be counselled that the ICSI procedure is safe even with epididymal sperm, but that we do not know the future outcomes like fertility and chances of diseases at later age.

For clinicians it is important to counsel their patients and inform them about these findings. Since we found that the risk of multiple pregnancies exceed the potential risks of ICSI with epididymal sperm, it stresses the importance of advocating single embryo transfer.

For policymakers in the Netherlands it is necessary to withdraw the moratorium and to see ICSI with epididymal sperm as a regular treatment in cases of obstructive azoospermia.

Recommendations for future research

Firstly, we will recommend to do research on larger groups and a longer follow up of the children born after IVF, ICSI with ejaculated or epididymal sperm, although the first results are reassuring. Special interests are late effects on health and fertility of these children. A similar follow up is recommended for the follow up of children born after ICSI with testicular sperm.

Secondly, we recommend concentration of new techniques in ART (like in vitro maturation, vitrification of oocytes, pre-implantation genetic screening), in specialized clinics with a good scientific evaluation of the treatment, outcome and follow up of the children.

Finally, we recommend a model for follow up of children born after new techniques in ART. This model must include: detection of DNA changes; questionnaires to collect data about the parents, pregnancy and children, developmental tests; a matched control group of natural conceived children; large study- and control groups; a blinded design and a long time of follow up. A model like this should be developed by the national and international professional organizations (Dutch Society of Reproductive Medicine (DSRM) and European Society of Human Reproduction and Embryology (ESHRE)). The latter might use its influence to call upon society to take its responsibility also with regard to proper health registries, with

concern of privacy.[33]

Conclusion

In conclusion, we showed in this thesis that ICSI with epididymal sperm is a safe treatment. Although there may be some theoretical and scientific indications for potential health risks for the children, we did not found increased numbers of congenital anomalies, stillbirth and developmental problems in a large clinical follow-up study.

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10

Chapter 11

Summary

Chapter 1

The birth of the first IVF-baby Louise Brown on July 25, 1978 was the start of a revolution for infertile couples worldwide. However, IVF could not help the infertile couples with severe male factor, which account for approximately half of the cases. The introduction of ICSI in 1992 enabled the management of severe male factor infertility with high success rates, even for men with azoospermia, ICSI with sperm retrieved at the level of the epididymis or testis is a possibility to father their own genetic progeny.

The use of IVF and ICSI with ejaculated and non-ejaculated sperm as well, has increased and its effectiveness has grown over time. Along with the success story, less attention is paid to safety and the potential risks and complications of this treatment. In the Netherlands, there were concerns about the health of the children born after ICSI with non-ejaculated sperm, referring to unknown risks of using aged (epididymal) or immature (testicular) sperm cells. This led to a national moratorium for the application of ICSI with non-ejaculated sperm in 1996. In 2001 the Dutch government agreed to start a new prospective and multicentre clinical study to confirm whether ICSI with epididymal sperm in case of an obstructive azoospermia can be used as a safe treatment.

This chapter describes the background (theoretical risks and complications of ICSI), the aim (follow-up of the children conceived after ICSI with ejaculated and especially with non-ejaculated sperm) and outline (literature searches, analyses of the results of the questionnaires and clinical studies of ICSI children) of this thesis and mentions the main questions.

Chapter 2

In this chapter we reviewed the literature about risks and complications of ICSI. There are theoretical risks of the female gamete, the male gamete and the process of genomic imprinting. These theoretical risks may result in fertilisation failure, abortion, birth defects, genetic abnormalities, developmental abnormalities and infertility in the offspring. The incidence of chromosomal abnormalities, including de novo abnormalities, is higher after ICSI than in the general population. This might be a result of the infertility per se rather than the ICSI technique. The incidence of congenital malformations might be slightly higher after ICSI, with special attention for children born after ICSI using epididymal or testicular sperm obtained from men with obstructive or non-obstructive azoospermia. The increased risk of congenital malformations seems also to be related to preterm and multiple births, so a twin pregnancy is regarded as a complication.

Risks for women by inducing ovulation or retrieving oocytes for ICSI are rare. Risks for men, after surgical sperm retrieval by epididymal sperm aspiration, or testicular sperm extraction in case of obstructive or non-obstructive azoospermia, are also rare.

Chapter 3

Pre-eclampsia affects 2–10% of all pregnancies and is a major cause of maternal and fetal morbidity and mortality. As compared with the general population, IVF pregnancies are associated with a 2.7-fold risk of pre-eclampsia.

In this chapter, we hypothesize that decreased ovarian reserve in women is associated with pre-eclampsia as a vascular complication in pregnancy. We conducted a retrospective case–control study and compared 41 cases with a history of pre-eclampsia to 82 matched controls without hypertension or (pre)eclampsia. All pregnancies were established after IVF or ICSI treatment. The 82 controls were matched according to the type of fertility treatment and several characteristics known to influence the risk of pre-eclampsia in pregnancy: the number of fetuses, parity, maternal age at the time of delivery, pre-pregnant BMI (kg/m^2), race and smoking.

The administered FSH units per follicle and per obtained oocyte were calculated, defined as the total amount of administered FSH divided by the number of follicles and the number of obtained oocytes, respectively. The condition of the neonates was evaluated as well.

We found that a higher amount of total administered FSH and FSH per day, together with a lower number of obtained oocytes during IVF treatment, were associated with an increased risk to pre-eclampsia in a subsequent pregnancy. The administered FSH per follicle and per obtained oocyte showed even stronger relationship, the latter having the best predictive value. The neonatal outcome was comparable between the groups.

We concluded that diminished responsiveness of the ovaries to FSH stimulation in an IVF cycle, reflecting decreased ovarian reserve, is associated with an increased risk of developing pre-eclampsia in a subsequent pregnancy. We speculated that reduced ovarian responsiveness also reflects diminished vascular reserve capacity, the latter giving rise to pregnancy associated vascular complications.

Chapter 4

Singletons conceived through IVF and ICSI have a lower birth weight than children conceived spontaneously. Whether the effect is limited to birth weight or reflects a general delay in growth is not yet known.

In this chapter we compared birth weight and longitudinal growth in the first 4 years of life of 347 IVF and 330 ICSI (with ejaculated sperm) term singletons (gestational age of ≥37 weeks) with those of 5059 term singletons of a national reference group.

The mean birth weights were calculated from the observed data, and the weights from 1 month to 4 years were calculated by using a linear mixed model. A linear regression model was developed to estimate the average weight curves in the two sex categories of the reference group, separately.

Our study showed that term IVF and ICSI singletons had a significantly lower birth weight than children from the national reference group. We also showed that the longitudinal growth of the IVF and ICSI children is comparable with the growth of natural conceived children and that the difference of birth weight was lost before the age of 4 years. The differences between the IVF and ICSI group were never statistically significant.

Chapter 5 and 6

In 2001 the Dutch government agreed to start a new prospective clinical study to confirm whether ICSI with epididymal sperm in case of an obstructive azoospermia can be used as a safe treatment. In chapter 5 and 6 we described the first clinical results of this study: firstly the results of PESA and testis biopsies (chapter 5) and secondly the clinical results of PESA-ICSI-treatment (chapter 6).

In our hospital PESA was carried out in 93 men with supposed obstructive azoospermia. From all men a testis biopsy was taken at the same time for the determination of the Johnsen score as a measurement of spermatogenesis. Epididymal motile sperm was retrieved from 76 patients (82%). In 73 of these patients the Johnsen score was higher than or equal to 8 and three had a score of 7.4. The average Johnsen score was statistically significant higher in cases when sperm was retrieved by PESA than in those cases when no sperm could be retrieved ($P < 0,001$).

In cases of an unexplained cause of azoospermia, there was a lower probability of retrieval of epididymal sperm than in those cases when the cause was known, but the difference between the two groups was not statistically significant.

In chapter 6 we described the first clinical results of the PESA-ICSI-treatment. In the first year, 31 couples started in total 44 cycles of ICSI with epididymal sperm retrieved by a PESA. There were 15 ongoing pregnancies (34% per started cycle). In one pregnancy, a chromosomal anomaly was diagnosed by amniocentesis, probably a de novo anomaly. This pregnancy was terminated. There were 17 children born, no congenital defects were found in any of them. There were no differences in percentage of ongoing pregnancies between the subgroups of cause of obstructive azoospermia or between the use of cryopreserved sperm and fresh sperm.

Chapter 7

In parallel with the advent of technological developments facilitating ART, especially of ICSI in cases of men suffering from severe oligozoospermia or azoospermia, there is a great concern about the biological safety. This concern is supported by the clinical observation that the frequency of congenital malformations is slightly elevated among ART-conceived children and a significantly higher rate of de novo chromosomal anomalies has been observed in ICSI-mediated offspring.

In this chapter we describe an explorative study, using tiling-resolution BAC array-mediated comparative genomic hybridization to investigate the incidence of de novo genomic copy number changes in a group of 12 ICSI children, compared with a control group of 30 naturally conceived children. In 6 of the 12 ICSI children, we found 10 apparently de novo 'same direction genomic copy number changes' (i.e. simultaneous copy number gain (or loss) with respect to both biological parents), notably losses. In statistically significant contrast, similar observations were encountered only six times in the control group in 5 of the 30 children. No significant correlation was found for 'same direction copy number changes' in children born after ICSI with sperm from a PESA and in children born after ICSI with ejaculated sperm from fathers suffering extreme OAT. None of the children had major or minor malformations, so the genomic changes do not seem to have phenotypic consequences.

Chapter 8

In this chapter a systematic review was carried out to investigate the karyotypes of fetuses, congenital anomalies and the follow-up of the children born after ICSI with epididymal or testicular sperm.

Because of the differences between the control groups, we analysed the studies, which described children born after epididymal sperm and after testicular sperm and children born after ejaculated sperm. We used the group with children born after ICSI with ejaculated sperm as the control group. The search strategy identified 1662 potentially relevant studies, but after exclusion, only eight articles were included in the systematic review. Two studies discussed karyotyping of fetuses, five studies were dealing with congenital anomalies and only one study was found with follow-up of the children.

There were no statistical differences in abnormal karyotypes, major malformations and follow-up of the children found in the studies we analysed, but it should be considered that the study groups were small and heterogenic with numerous potential biases.

Chapter 9

In this chapter we evaluate the safety of ICSI with epididymal sperm, by comparing children born after ICSI-treatment with epididymal sperm and children conceived after IVF and ICSI with ejaculated sperm. Additionally, the results of a multidisciplinary, multicentre follow-up of the children conceived with epididymal sperm at two years of age are described.

This follow-up study included 378 children after ICSI with epididymal sperm (PESA group) and a control group of 1192 IVF and 1126 ICSI (with ejaculated sperm) children, all with a gestational age of 20 weeks or more. Questionnaires were sent at birth, at one year and at four years of age, collecting data on parental, pregnancy and child factors, including gestational age, mode of delivery, birth weight, presence or absence of malformations and neonatal problems. 148 children born after ICSI with epididymal sperm were assessed at two years of age for motor performance, mental- and language development and compared with the Dutch norms.

Children born after ICSI with epididymal sperm showed no increased risks for stillbirths, total deaths and malformations. They also did not differ from IVF and ICSI (with ejaculated sperm) children in gender rate, birth weight and gestational age. The minor malformations rate in the PESA group was significantly lower than in the control groups. The mental Bayley score was significantly higher for PESA singletons and parents reported less behavioural problems in the PESA group than the Dutch reference group. The scores for syntactic development and lexical development for the PESA singletons were significantly better than the Dutch standards.

Chapter 10

In this chapter the answers on the main questions are mentioned and discussed. The most relevant methodological issues are reviewed and the implications for the different stakeholders and recommendations for future research are given.

Theoretical risks of ICSI (especially with non-ejaculated sperm) for men, women and children are mentioned and discussed. Although there remain some concerns about the findings of lower birth weight of IVF and ICSI children compared to naturally conceived singletons, these differences disappeared before the age of 4 years. There were statistical significant more 'same direction copy number changes' of DNA in ICSI children (with ejaculated and epididymal sperm) compared to the control group, but there were not more congenital malformations found in ICSI children conceived with ejaculated or epididymal sperm compared to IVF children. In the literature and in our clinical follow up study, there were not more concerns about children born after ICSI with epididymal sperm compared to ICSI with ejaculated sperm or after IVF. There are still concerns about the mortality and morbidity of multiples, which are seen more often after IVF and ICSI treatment.

Samenvatting
Publications
Dankwoord
Curriculum Vitae
PhD theses Human Reproduction

Samenvatting

Hoofdstuk 1

De geboorte van de eerste IVF-baby Louise Brown op 25 juli 1978, was het begin van een wereldwijde revolutie voor infertiele paren. Helaas was IVF niet de oplossing voor paren met een ernstige mannelijke factor, wat ongeveer de helft van de indicaties betreft. Voor deze paren maakte de introductie van ICSI in 1992 het mogelijk om genetisch eigen kinderen te krijgen, zelfs voor mannen met een azoöspermie, waarbij ICSI wordt verricht met zaadcellen verkregen uit de epididymis of testis.

In de afgelopen jaren zijn het aantal IVF en ICSI behandelingen, zowel met geëjaculeerd als met niet-geëjaculeerd zaad, toegenomen en is de effectiviteit verbeterd. Naast deze goede resultaten is er minder aandacht besteed aan de veiligheid en potentiële risico's en complicaties van deze behandelingen. In Nederland was men vooral bezorgd over de gezondheid van de kinderen, die ontstaan waren na ICSI met niet-geëjaculeerd zaad, vooral door de onbekende risico's van het gebruik van oudere (epididymale) of niet rijpe (testiculaire) zaadcellen. Dit heeft geleid tot een nationaal moratorium in 1996, waarbij het verboden was om ICSI te verrichten met niet-geëjaculeerd zaad. Na geruststellende onderzoeken uit het buitenland, stemde de Nederlandse regering in 2001 toe met het gedeeltelijk opheffen van het moratorium onder voorwaarde dat alleen ICSI verricht zou worden met epididymaal zaad in gevallen van een obstructieve azoöspermie. Tevens zou er een goede follow-up moeten plaats vinden van de kinderen die uit deze behandeling geboren zouden worden om te bewijzen dat het een veilige methode is.

Dit hoofdstuk beschrijft de achtergrond (theoretische risico's en complicaties van ICSI), het doel (follow-up van de kinderen ontstaan na ICSI met geëjaculeerd en met name ook na niet-geëjaculeerd zaad) en de opzet (literatuur studies, analyses van de resultaten van de vragenlijsten en klinische onderzoeken van de ICSI kinderen) van dit proefschrift en benoemt de hoofdvragen.

Hoofdstuk 2

In dit hoofdstuk wordt besproken wat er in de literatuur is beschreven over de risico's en complicaties van ICSI. Zo zijn er theoretische risico's van de vrouwelijke gameet (eicel), de mannelijke gameet (zaadcel) en het proces van inprenting van het genoom. Deze theoretische risico's kunnen resulteren in het ontbreken van bevruchting, miskramen, aangeboren afwijkingen, genetische afwijkingen, ontwikkelingsstoornissen en infertiliteit bij het nageslacht. De incidentie van chromosomale afwijkingen, inclusief nieuwe afwijkingen, is hoger na ICSI dan in de algemene populatie. Dit kan een resultaat zijn van de oorzaak van de infertiliteit of van de ICSI procedure zelf. Het vóórkomen van aangeboren afwijkingen zou mogelijk wat hoger zijn bij kinderen geboren na ICSI, waarbij vooral aandacht is voor de kinderen ontstaan na ICSI met epididymaal of testiculair zaad. Dit verhoogde risico op aangeboren afwijkingen zou ook kunnen komen door het hogere aantal vroeggeboortes bij meerlingen, zodat ook een tweelingzwangerschap als complicatie gezien moet worden.

Risico's voor de vrouw door de hormoonstimulatie en het verkrijgen van de eicellen, zijn zeldzaam. Ook voor de man zijn er weinig risico's na een chirurgische ingreep, waarbij zaad wordt verkregen uit de epididymis of testis in het geval van respectievelijk een obstructieve of niet-obstructieve azoöspermie.

Hoofdstuk 3

In 2-10% van alle zwangerschappen treedt pre-eclampsie op als complicatie en het is tevens één van de hoofdoorzaken van ziekte en sterfte bij moeder en kind. Vergeleken bij de algehele populatie komt pre-eclampsie 2,7 maal zoveel voor bij IVF-zwangerschappen.

In dit hoofdstuk hebben we de aanname gedaan dat verminderde ovariële reserve bij de vrouw geassocieerd is met pre-eclampsie als een vasculaire complicatie tijdens de zwangerschap. We hebben een retrospectieve case-control studie uitgevoerd waarbij we 41 zwangere vrouwen met pre-eclampsie in de voorgeschiedenis vergeleken met 82 vergelijkbare controles zonder hypertensie of (pre)eclampsie. Al deze zwangerschappen waren ontstaan na een IVF of ICSI behandeling. De 82 controles waren gematched op soort fertiliteitsbehandeling en op verschillende karakteristieken die invloed kunnen hebben op het risico van pre-eclampsie in de zwangerschap: het aantal foetussen, pariteit, leeftijd moeder op dag van bevalling, BMI (kg/m^2), ras en rookgedrag.

Het aantal toegediende eenheden FSH per follikel en per verkregen oöcyt werd berekend, gedefinieerd als de totale hoeveelheid van toegediende eenheden FSH gedeeld door het aantal follikels respectievelijk het aantal verkregen oöcyten. De gezondheid van de pasgeborene werd eveneens geëvalueerd.

We vonden dat een hogere hoeveelheid van totaal toegediend FSH en FSH per dag samen met een lager aantal verkregen oöcyten bij een IVF behandeling, waren geassocieerd met een hogere kans op pre-eclampsie in een daaropvolgende zwangerschap. Het aantal toegediende eenheden FSH per follikel en per verkregen oöcyt gaf zelfs een betere relatie, waarbij de laatst genoemde een betere voorspellende waarde had. De gezondheid van de pasgeborenen was vergelijkbaar tussen de groepen.

We concludeerden dat verminderde respons van de ovaria op FSH tijdens een IVF behandeling een weergave is van verminderde ovariële reserve en geassocieerd is met een verhoogd risico op het ontwikkelen van pre-eclampsie in een daaropvolgende zwangerschap. We speculeerden dat verminderde ovariële respons een weergave is van verminderde vasculaire reserve capaciteit, welke laatste een verhoging kan geven op vasculaire complicaties die geassocieerd zijn met een zwangerschap.

Hoofdstuk 4

Eenlingen die ontstaan zijn na een IVF of ICSI behandeling hebben een lager geboortegewicht dan kinderen die spontaan zijn ontstaan. Of dit effect beperkt is tot het geboortegewicht of ook invloed heeft op een algehele vertraging in groei, is niet bekend.

In dit hoofdstuk vergelijken we het geboortegewicht en de longitudinale groei in de eerste vier levensjaren van 347 IVF en 330 ICSI (met geëjaculeerd zaad) aterm geboren eenlingen (zwangerschapsduur ≥ 37 weken) met die van 5059 aterm geboren eenlingen uit een nationale referentiegroep.

Het gemiddelde geboortegewicht werd berekend uit de geobserveerde data en de gewichten van 1 maand tot 4 jaar werden berekend met behulp van een lineair mixed model. Een lineair regressie model was ontwikkeld om de gemiddelde groeilijnen te schatten voor de twee afzonderlijke geslachten van de referentiegroep.

Onze studie liet zien dat aterm geboren IVF en ICSI eenlingen een significant lager geboortegewicht hadden dan de kinderen uit de nationale referentiegroep. Tevens toonden we aan dat de longitudinale groei van IVF en ICSI kinderen vergelijkbaar is met die van natuurlijk verwekte kinderen en dat het verschil van geboortegewicht reeds verdwenen was voor de leeftijd van 4 jaar. De verschillen tussen de IVF en ICSI groep waren nergens significant.

Hoofdstuk 5 en 6

In 2001 stemde de Nederlandse regering in met het starten van een nieuwe prospectieve klinische studie om aan te tonen dat ICSI met epididymaal zaad, in het geval van een obstructieve azoöspermie, een veilige behandeling is. In hoofdstuk 5 en 6 beschrijven we de eerste klinische resultaten van deze studie: ten eerste de resultaten van de PESA en de testis biopsie (hoofdstuk 5) en ten tweede de klinische resultaten van de PESA/ICSI-behandeling (hoofdstuk 6).

In ons ziekenhuis werd een PESA verricht bij 93 mannen met een vermeende obstructieve azoöspermie. Tegelijkertijd werd bij al deze mannen een testis biopt afgenomen om een Johnsen score te bepalen als maat voor de spermatogenese. Epididymaal beweeglijke zaadcellen werden verkregen bij 76 patiënten (82%). Bij 73 van deze patiënten was de Johnsen score hoger of gelijk aan 8 en drie hadden een score van 7,4. De gemiddelde Johnsen score was statistisch significant hoger in de gevallen waarbij zaadcellen was verkregen bij de PESA, dan bij de gevallen waarbij geen zaadcellen werden gevonden ($P < 0,001$).

Bij de mannen zonder duidelijke oorzaak van de azoöspermie was een lagere kans op het verkrijgen van epididymaal zaad, dan bij de mannen waarvan de oorzaak wel bekend was. Echter, het verschil tussen de groepen was niet statistisch significant.

In hoofdstuk 6 worden de eerste klinische resultaten van de PESA/ICSI-behandeling beschreven. In het eerste jaar werden bij 31 paren in totaal 44 cycli opgestart voor een ICSI behandeling met epididymaal zaad, verkregen bij een PESA. Er traden 15 doorgaande zwangerschappen op (34% per gestarte cyclus). Bij één zwangerschap werd bij vruchtwaterpunctie een chromosomale afwijking gediagnosticeerd, waarschijnlijk een nieuw ontstane afwijking. Deze zwangerschap werd voortijdig afgebroken. Er werden 17 kinderen geboren, bij geen van hen werd een aangeboren

afwijking gevonden. Er waren geen verschillen in percentage van doorgaande zwangerschappen tussen de subgroepen naar oorzaak van de azoöspermie en ook niet tussen het gebruik van ingevroren zaad of vers zaad.

Hoofdstuk 7
Gelijktijdig met de voortschrijding van technologische ontwikkelingen rond ART, speciaal bij ICSI in het geval van mannen met ernstige oligospermie of azoöspermie, zijn er grote zorgen rond de biologische veiligheid. Deze zorgen worden gesteund door de klinische observatie dat de frequentie van aangeboren afwijkingen licht verhoogd is bij kinderen geboren na ART en dat er een statistisch verhoogd aantal nieuw ontstane chromosomale afwijkingen zijn geconstateerd bij ICSI kinderen.

In dit hoofdstuk beschrijven we een verkennende studie waarin we met een hoge resolutie Bacteriële Artificiële Chromosomen array de incidentie van nieuw ontstane genomische kopie aantal veranderingen in 12 ICSI kinderen hebben onderzocht en deze hebben vergeleken met een controlegroep van 30 natuurlijk verwekte kinderen. In 6 van de 12 ICSI kinderen vonden we in totaal 10 nieuwe genomische kopie aantal veranderingen, waarbij het kind een toename (of juist afname) in kopie aantal liet zien ten opzichte zowel de biologische vader als de moeder. Het betroffen hier vooral afnames in kopie aantal. Er was een statistisch significant verschil met de controle groep, waarbij slechts 6 vergelijkbare observaties waren bij 5 van de 30 kinderen. Er waren geen significante verschillen gevonden voor genomische kopie aantal veranderingen tussen kinderen geboren na ICSI met zaad van een PESA en kinderen geboren na ICSI met geëjaculeerd zaad van vaders met een extreme OAT. Geen van de kinderen had grote of kleine afwijkingen, dus de genomische veranderingen lijken geen fenotypische consequenties te hebben.

Hoofdstuk 8
In dit hoofdstuk wordt een systematische literatuurstudie beschreven naar de karyotypering van foetussen, aangeboren afwijkingen en de follow-up van kinderen geboren na ICSI met epididymaal of testiculair zaad.

Vanwege de verschillen tussen de controle groepen, analyseerden we alleen de studies die kinderen beschreven die ontstaan waren na gebruik van epididymaal zaad en na testiculair zaad en kinderen geboren na gebruik van geëjaculeerd zaad. De groep kinderen geboren na ICSI met geëjaculeerd zaad gebruikten we als controle groep. De zoekstrategie leverde 1662 potentieel relevante studies op, maar na exclusie waren slechts acht artikelen bruikbaar voor deze systematische literatuurstudie. Twee studies beschreven de karyotypering van foetussen, vijf studies gingen over aangeboren afwijkingen en slechts één was gevonden over de follow-up van de kinderen.

Er waren geen statistische verschillen gevonden in de studies die we analyseerden betreffende abnormale karyotypes, grote aangeboren afwijkingen en follow-up van de kinderen, hierbij echter wel in aanmerking genomen dat de studiegroepen klein en heterogeen waren met talrijke potentiële bias.

Hoofdstuk 9
In dit hoofdstuk evalueren wij de veiligheid van ICSI met epididymaal zaad, door het vergelijken van kinderen geboren na een ICSI behandeling met epididymaal zaad en kinderen ontstaan na IVF en ICSI met geëjaculeerd zaad. Aanvullend worden de resultaten beschreven van een multidisciplinaire, multicentrum follow-up op tweejarige leeftijd van de kinderen ontstaan na epididymaal zaad.

Deze follow-up studie bestaat uit 378 kinderen geboren na ICSI met epididymaal zaad (PESA groep) en een controle groep van 1192 IVF en 1126 ICSI (met geëjaculeerd zaad) kinderen, allen geboren na een zwangerschapsduur van 20 weken of meer. Er werden vragenlijsten gestuurd bij de geboorte, bij één jaar en op 4 jarige leeftijd. Hierbij werden gegevens gevraagd betreffende de ouders, zwangerschap en het kind, inclusief zwangerschapsduur, bevallingsmechanisme, geboortegewicht, aan- of afwezigheid van aangeboren afwijkingen en neonatale problemen. Op 2-jarige leeftijd werden 148 kinderen, die ontstaan waren na ICSI met epididymaal zaad, getest op motorische-, mentale- en spraak/taal-ontwikkeling en vergeleken met de Nederlandse norm.

Kinderen geboren na ICSI met epididymaal zaad lieten geen verhoogd risico zien voor het aantal doodgeborenen, het totaal aantal overleden kinderen en aangeboren afwijkingen. Ook was er geen verschil met de IVF en ICSI (met geëjaculeerd zaad) kinderen betreffende ratio jongens/ meisjes, geboortegewicht en zwangerschapsduur. Het percentage kleine aangeboren afwijkingen was bij de PESA groep significant lager dan in de controle groepen. De mentale Bayley score was significant hoger voor de PESA eenlingen en de ouders meldden minder gedragsproblemen bij de PESA groep dan de Nederlandse referentiegroep. De scores voor de taal- en spraak ontwikkeling van de PESA eenlingen was significant beter dan de Nederlandse standaard.

Hoofdstuk 10
In dit hoofdstuk worden de antwoorden op de hoofdvragen gegeven en bediscussieerd. De meest relevante methodologische problemen worden besproken en de consequenties voor de verschillende belanghebbenden en aanbevelingen voor de toekomst worden gegeven.

Theoretische risico's van ICSI (vooral met niet-geëjaculeerd zaad) voor mannen, vrouwen en kinderen worden benoemd en bediscussieerd. Ook al blijven er zorgen over de bevinding van het lagere geboortegewicht van IVF en ICSI kinderen vergeleken met natuurlijk verwekte eenlingen, deze verschillen zijn verdwenen voor de 4-jarige leeftijd. Er waren significant meer genomische kopie aantal veranderingen van het DNA bij ICSI kinderen (zowel met geëjaculeerd als met epididymaal zaad) vergeleken met de controle groep, maar er waren niet meer aangeboren afwijkingen gevonden bij ICSI kinderen ontstaan met geëjaculeerd of epididymaal zaad vergeleken met IVF kinderen. In zowel de literatuur als in onze klinische follow-up studie waren er niet meer zorgen voor de kinderen geboren na ICSI met epididymaal zaad vergeleken met ICSI met geëjaculeerd zaad of na IVF. Wel zijn er nog steeds zorgen over de ziekte en sterfte van meerlingen, welke nog steeds vaak gezien worden na een IVF of ICSI behandeling.

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Curriculum Vitae

Gwendolyn Woldringh werd op 11 augustus 1959 geboren in Alkmaar. Na enkele omzwervingen via Italië en Bennekom (waar zij de basisschool doorliep), woonde zij in de bossen bij Wageningen. Zij ging naar het Heldring College in Zetten: elke dag met de fiets en later op de brommer, met het pontje over de Rijn. In 1977 behaalde zij haar VWO diploma.

Na een jaar uitgeloot te zijn (waarin "van alles en nog wat" werd gedaan), mocht zij in 1978 in Nijmegen beginnen met de studie waar ze al lang naar uit had gekeken: geneeskunde. Van kleins af aan had ze in haar hoofd om "kinderdok" te worden, maar tijdens haar co-schappen kwam ze er achter dat dit toch niet helemaal was wat ze zocht.

Twee dagen na haar afstuderen in maart 1986, startte zij haar (naar later bleek) gevarieerde carrière, als AGNIO op de afdeling kinderchirurgie in het St Radboud ziekenhuis in Nijmegen. Dit duurde slechts 2 en een halve maand, echter binnen hetzelfde ziekenhuis kon ze gelijk doorstromen naar de afdeling algemene heelkunde. Ook hier was ze AGNIO, maar nu 2 en een half jaar. Dit bleek een vak, waar ze veel voldoening uithaalde, zodat ze in 1988 probeerde om in opleiding te komen voor chirurgie. Helaas werd ze hier niet voor aangenomen en aangezien in die tijd maar één keer meegedaan kon worden aan een opleidingsronde, viel haar droom om ooit kinderchirurg te worden in duigen. Tevens werd zij 2 maal uitgeloot voor de huisartsenopleiding, zodat de knop om gezet moest worden. Dit lukte door gelijk aan de slag te kunnen als docent anatomie en fysiologie bij de in-service opleiding van het St Radboud ziekenhuis voor A-verpleegkundigen en als docent chirurgie bij de specialisatie voor eerste-hulp verpleegkundigen. Gelijktijdig kon ze gaan waarnemen (en na enige tijd ook in vast dienstverband) als consultatiebureau-arts in Nijmegen en omgeving.

Naast deze werkzaamheden had ze samen met haar man Joep (met wie zij in 1985 getrouwd is) het geluk om de trotse ouders te mogen worden van vier prachtige dochters: Pippa, Mytte, Dedde en Birre. Op het moment dat de jongste ruim een jaar was en ze al 10 jaar werkzaam was op het consultatiebureau, begon het te kriebelen en werd er naar een nieuwe uitdaging gezocht. Dit werd gevonden in wederom het St Radboud ziekenhuis, maar dan als IVF arts. Vanaf het begin (maart 1999) was ze bezig met het verzamelen van data over de gezondheid van de

IVF en ICSI kinderen en later ook van de PESA kinderen. Na de eerste publicatie in december 2003 over de eerste resultaten van de ICSI/ PESA behandelingen, werd er daadwerkelijk een plan richting promotie gesmeed. Dit proefschrift is het uiteindelijke resultaat hiervan!

PhD theses Human Reproduction NCEBP (2000-2011)

1. *07-02-2000* **Els van der Molen**
   Disturbed homocysteine metabolism endothelial dysfunction and placental vasculopathy

2. *29-06-2000* **Willianne Nelen**
   Risk factors for recurrent early pregnancy loss. Hyperhomocysteinaemia, thrombophilia and impaired detoxification

3. *05-09-2000* **Ina Beerendonk**
   Sodium and ovarian hyperstimulation. Some clinical and psychological aspects

4. *04-12-2000* **Anne-Marie van Cappellen van Walsum**
   Cerebral metabolism of hypoxic fetal sheep by NMR spectroscopy

5. *18-12-2000* **Friso Delemarre**
   Vascular aspects of human pregnancy. Clinical studies on sodium restriction and angiotensin infusion

6. *10-01-2001* **Way Yee Wong**
   Male factor subfertility. The impact of lifestyle and nutritional factors

7. *05-06-2001* **Petra Zusterzeel**
   Biotransformation enzymes and oxidative stress in preeclampsia

8. *05-10-2001* **Cathelijne van Heteren**
   Development of habituation and memory in the human fetus

9. *10-10-2001* **Michael Gaytant**
   Cytomegalovirus and herpes simplex virus infections in pregnancy

10. *25-01-2002* **Ron van Golde**
    Male subfertility and genetics

11. *21-05-2002* **Tanja de Galan-Roosen**
    Perinatal Mortality

12. *08-01-2003* **Maarten Raijmakers**
    Oxidative stress and detoxification in reproduction with emphasis on glutathione and preeclampsia

13. *18-02-2003* **Sabine de Weerd**
    Preconception counselling. Screening & periconceptional health

14. *22-4-2003* **Iris van Rooij**
Etiology of Orofacial Clefts. Gene-environment interactions and folate

15. *17-12-2003* **Chris Verhaak**
Emotional impact of unsuccessful fertility treatment in women

16. *14-01-2004* **Liliana Ramos**
The quality of epididymal sperm in azoospermia

17. *04-10-2004* **Pascal Groenen**
Nutritional and environmental factors in human spina bifida. An emphasis on myo-inositol

18. *24-11-2004* **Tanya Bisseling**
Placental function in maternal disease. Ex vivo assessment of foetoplacental vascular function and transport in diabetes and preeclampsia

19. *15-12-2004* **Eva Maria Roes**
Oxidant-antioxidant balance and maternal health in preeclampsia and HELLP syndroom

20. *01-06-2005* **Marieke Rijnsaardt-Lukassen**
Single Embryo Transfer: clinical and immunological aspects

21. *10-11-2005* **Ingrid Krapels**
The etiology of orofacial clefts. An emphasis on lifestyle and nutrition other than folate

22. *14-06-2006* **Reini Bretveld**
Fertility among greenhouse workers

23. *09-11-2006* **Jesper Smeenk**
Stress and IVF. Clinical consequences

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Human subfertility: explorative studies on some pathophysiologic factors in semen and follicular fluid

25. *01-11-2007* **Alwin Derijck**
The transmission of chromatin and DNA lesions by sperm and their fate in de zygote (1)

26. *01-11-2007* **Godfried van der Heijden**
The transmission of chromatin and DNA lesions by sperm and their fate in de zygote (2)

27. *03-12-2007* **Kirsten Kluivers**
On the measurement of recovery following hysterectomy

28. *10-12-2007* **René Kok**
Proton Magnetic Resonance Spectroscopy of Human fetal brain

29. *10-12-2008* **Trudie Gerrits**
Clinical encounters: Dynamics of patient-centred practices in a Dutch fertility clinic

30. *12-12-2008* **Wouter Tuil**
IVF and Internet

31. *06-03-2009* **Ineke Krabbendam**
Venous reserve capacity & autonomic function in formerly preeclamtic women

32. *03-09-2009* **Arno van Peperstraten**
Implementation of single embryo transfer

33. *10-03-2010* **Suzanne Broekhuis**
Dynamic MR imaging in female pelvic floor disorders

34. *12-03-2010* **Bea Lintsen**
IVF in the Netherlands: success rates, lifestyle, psychological factors and costs

35. *21-04-2010* **Selma Mourad**
Improving fertility care: the role of guidelines, quality indicators and patients

36. *24-02-2011* **Monique Brandes**
Observational studies in reproductive medicine

37. *22-06-2011* **Marian Spath**
Risk estimate for fragile X-associated primary ovarian insufficiency: genetic, environmental and reproductive factors

38. *30-06-2011* **Inge van Empel**
Patient-centredness in fertility care

39. *18-11-2011* **Gwendolyn Woldringh**
ICSI children. Follow-up after ICSI with ejaculated or non-ejaculated sperm